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OXYGEN ISOTOPE COMPOSITION OF SULPHATE PRODUCED DURING BACTERIAL OXIDATION OF METAL SULPHIDES: DEPENDENCE UPON O_2 PRESSURE AND OXGYEN ISOTOPE COMPOSITIONS OF H_2O AND O_2

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EXECUTIVE SUMMARY

The oxygen isotope composition of SO_4^{2-} produced during oxidation of metal sulphides by T. ferrooxidans under a variety of conditions, was examined. Reaction variables included different additions of $^{18}O_2$ and $H_2{}^{18}O$ and varying O_2 pressures. Some reactions which are considered to be part of the overall mechanism, were also studied.

During atmospheric oxidation of pentlandite in H_2O of different $\delta^{18}O$ values, it was found that

$$\delta^{18}O\ (SO_4^{2-}) = (0.74\pm0.01)\delta^{18}O(H_2O) + (9.0\pm3.3) \quad r^2 = 1.0$$

The slope is consistent with those previously observed for FeS_2 and pentlandite oxidation under similar conditions.

A series of closed container experiments proved difficult to interpret but it was concluded that (1) the oxidation rate of FeS_2 by T. ferrooxidans was increased slightly with higher O_2 pressures (2) the oxygen isotope composition of SO_4^{2-} is influenced by O_2 pressure, and (3) there are significant kinetic isotope effects during O_2 incorporation. The results are relevant to mineral sulphide oxidation in O_2 limited subsurface environments and in turn, global sulphur cycling.

One highlight of the study was that H_2O and O_2 which do not normally exchange oxygen isotopes, did so readily during pentlandite oxidation by T. ferrooxidans. This has profound mechanistic and geochemical significance.

During the conversion of SSO_3^{2-} to SO_3^{2-} with or without the presence of rhodanese, there was no observable exchange of oxygen isotopes between O_2 and H_2O.

During oxidation of SO_3^{2-} to SO_4^{2-}, oxygen was incorporated from H_2O into SO_4^{2-} with or without oxidase present. The slope of the plot of $\delta^{18}O$ (SO_4^{2-}) versus $\delta^{18}O$ (H_2O), 0.57, was close to that realized in the overall mineral sulphide oxidation. Therefore, the SO_3^{2-} oxidation step is possibly the dominant determiner of the oxygen isotope composition of SO_4^{2-} produced in the overall reaction. This observation is also relevant to previously published work on oxygen isotope behavior during SO_4^{2-} reduction.

The transfer of oxygen from O_2 to SO_4^{2-} during SO_3^{2-} oxidation was also demonstrated using different $^{18}O_2$ additions. Another landmark observation was that the product SO_4^{2-}acquires about twice as much ^{18}O when oxidase was present than when it was absent. This means that the ratio of oxygen derived from O_2 to that obtained from H_2O was much higher when oxygenase participated. This finding and the better understanding of the role of enzymes generally, is desirable from the viewpoint of enhancing or retarding mineral sulphide oxidation. It should also have relevance in other research fields such as the medical sciences.

Résumé administratif

La composition isotopique en oxygène de SO_4^{2-} produit au cours de l'oxydation de sulfures métalliques par T. ferrooxidans dans différentes conditions a été éxaminée. Parmi les paramètres étudiés, on a fait varier la quantité de $^{18}O_2$ et de $H_2{}^{18}O$ ajoutée ainsi que la pression de O_2. Certaines réactions mises en jeu dans le mécanisme global ont été également étudiées.

Lors de l'oxydation atmosphérique de la pentlandite dans H_2O contenant différentes valeurs de $\delta^{18}O$, l'équation suivante a été obtenue:

$$\delta^{18}O\ (SO_4^{2-}) = (0.74\pm0.01)\delta^{18}O(H_2O) + (9.0\pm3.3) \quad r^2 = 1.0$$

La pente est conforme aux valeurs calculées pour l'oxydation de FeS_2 et de la pentlandite dans les mêmes conditions.

Les résultats d'une série d'expériences en réacteur fermé ont été difficiles à interpréter. Cependant, il a été conclu que (1) le taux d'oxydation de FeS_2 par T. ferrooxidans a été légèrement augmenté aux plus hautes pressions d'O_2 (2) la composition isotopique en oxygène de SO_4^{2-} est influencée par la pression de O_2 et (3) les cinétiques d'incorporation de O_2 sont dépendentes de la composition isotopique de O_2. Les résultats sont applicables à l'oxydation des minéraux sulfurés dans des environnements de sub-surface à faible teneur en O_2, et par là-même, au cycle global du sulfure.

Un des points importants de l'étude a été que H_2O et O_2, qui normallement n'échangent par leur oxygène isotopique,

l'ont effectivement échangé au cours de l'oxydation de la pentlandite par T. ferrooxidans. Ceci à une grande importance pour la compréhension des mécanismes de réaction et des processus géochimiques.

Au cours de la transformation de $S_2O_3^{2-}$ en SO_3^{2-} en présence ou non de rhodanase, aucun échange des isotopes de l'oxygène entre O_2 et H_2O n'a pu être observé.

Au cours de l'oxydation de SO_3^{2-} en SO_4^{2-}, l'oxygène est passé de H_2O dans SO_4^{2-} en présence ou non d'oxydase. La pente de la courbe $\delta^{18}O$ (SO_4^{2-}) en fonction de $\delta^{18}O$ (H_2O), égale à 0.57, est proche de celle obtenue lors de l'oxydation globale des minéraux sulfurés. Par conséquent, il se peut que l'étape d'oxydation de SO_3^{2-} soit l'étape déterminante de la composition isotopique en oxygène de SO_4^{2-} produit lors de la réaction globale. Cette observation est en accord avec les travaux déjà publiés sur le comportement des isotopes de l'oxygène au cours de la réduction de SO_4^{2-}.

L'addition de différentes quantités de $^{18}O_2$ a permis de mettre en évidence le transfer d'oxygène de O_2 vers SO_4^{2-}. Une autre observation intéressante a été que SO_4^{2-} incorpore deux fois plus de ^{18}O en présence d'oxydase. Cela signifie que le rapport entre l'oxygène provenant de O_2 et celui obtenu à partir de H_2O a été beaucoup plus élevé en présence d'oxygénase. Cette découverte ainsi que la meilleure compréhension du rôle des enzymes plus généralement, est fondamentale si l'on veut accélérer ou retarder l'oxydation des sulfures minéraux. Ces résultats pourraient également présenter un intérêt dàutres domaines de la recherche tels les sciences médicales.

INTRODUCTION

The oxygen isotope composition of sulphate produced during sulphide oxidation is highly variable. The mechanism is a combination "oxidation-hydrolysis" in which oxygen can be incorporated from two sources, O_2 and H_2O molecules (cf Lloyd, 1968; Taylor et al., 1984; van Everdingen and Krouse, 1985).

Much controversy exists in the literature as to the detailed mechanism. It embraces questions such as the proportions of oxygen derived from O_2 and H_2O, kinetic isotope effects (i.e. different reaction rates for ^{16}O and ^{18}O) during incorporation, and exchange of oxygen atoms among O_2, H_2O and reaction intermediates.

Prior to 1987, experiments were invariably conducted with pyrite, FeS_2. In that year, the author participated in a series of experiments with R.G.L. McCready and his colleagues at EMR. In order to better understand the bacterial oxidation mechanisms, the following novel features were incorporated into the investigation.

1. Bacterial oxidations were carried out in waters of various oxygen isotope compositions in order to ascertain how well the oxygen isotope composition of the product sulphate correlated to that of the water.
2. The experiments were maintained for up to 5 weeks with weekly sampling.

3. In addition to sulphate, the isotope composition of the water was monitored since kinetic isotope effects accompany evaporation.
4. Whereas previous reports were confined to pyrite, other metal sulphides (sphalerite, pentlandite, chalcopyrite, and mixed sulphide ore) were examined to see if the oxygen isotope composition of the sulphate was influenced by the nature of the reactant sulphide.

The results of these experiments were summarized by Krouse (1988). In all cases, it was found that the oxygen isotope composition of the sulphate correlated strongly with the water according to linear equations of the form

$$\delta^{18}O\ (SO_4^{2-}) = m\delta^{18}O\ (H_2O) + b\ *$$

The value of slope, m, ranged from 0.7 for pyrite and pentlandite, through 0.6 for sphalerite, down to 0.37 for chalcopyrite. In the latter experiments, there was substantial background carry over from the inoculum. Although corrections could be made for SO_4^{2-} transfer during inoculation, the effects of carry over of intermediates of the oxidation reaction were difficult to evaluate.

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* $\delta^{18}O = \left\{ \frac{[^{18}O/^{16}O]_{SAMPLE}}{[^{18}O/^{16}O]_{SMOW}} - 1 \right\} \times 10^3$ where $[^{18}O/^{16}O]$ is the ratio of the numbers of ^{18}O to ^{16}O atoms in a sample and SMOW (Standard Mean Ocean Water) is an internationally defined standard for reporting oxygen isotope abundances.

It was clear that, the participation of the water molecule in the reaction was substantial but the extent to which it was by direct reaction or isotope exchange with reaction intermediates, was still debatable. To resolve these alternatives, further experiments were designed which are the subject of this report.

Unique features incorporated in the current experiments were

(1) Experiments were conducted with ^{18}O-enriched O_2 as well as H_2O.

(2) Experiments were conducted at different O_2 pressures.

(3) Experiments were conducted with postulated intermediates of the oxidation reaction with or without enzymes.

MATERIALS AND METHODS

A Hudson Bay Mining and Smelting (HBM & S) ore sample was obtained from Flin Flon, Manitoba and pretreated with 2.0 N HCl in order to remove calcite and oxidation products. Isotopically enriched O_2 (96.5 atom % ^{18}O) was obtained from MSD Isotopes, Montreal, Canada.

The T. ferrooxidans isolate (EL-5) was isolated from a mine water sample (McCready et al., 1986). The inocula were maintained on the HBM & S ore (6 g ore + 100 mL autotrophic medium) in 250 mL Erlenmeyer flasks at 30°C on a rotary shaker (150 rpm). The autotrophic medium used during this study was a sulphate free medium of the following composition: K_2HPO_4 0.1 mM; $MgCl_2$ 0.5mM; NH_4Cl 1.0 mM, adjusted to pH 2.5.

At the end of a given experiment, the culture supernatant was filtered through Whatman #42 filter paper. Some of the culture supernatant was saved for mass spectrometry and the remainder was used for sulphate determination. Two mL of concentrated HCl was added to the aliquot taken for sulphate analysis, and the solution was heated to a boil. Then 10 mL of 10% w/v $BaCl_2$ was added to the acidified solution and the precipitated barium sulphate was allowed to settle overnight. The barium sulphate was filtered and collected on a 0.45µ Millipore filter, washed with 100 mL of 1 N HCl, 500 mL of deionized water and dried for 24 h at 60°C. The dried barium sulphate was weighed and transferred to small vials for subsequent ^{18}O determinations.

For oxygen isotope analyses, $BaSO_4$ was reduced by graphite in platinum boats through which currents of the order of 20A were passed. Both CO_2 and CO were produced in this high temperature reaction. The CO was converted to CO_2 using a high voltage discharge unit and a liquid N_2 bath. The apparatus is a variation of that described by Sakai and Krouse (1971).

The oxygen isotope composition of O_2 was determined on CO_2 prepared by passing O_2 over a small spectrographically pure graphite rod, wrapped in platinum wire, at 900°C. This technique posed some problems with memory effects. It was also difficult to prevent atmospheric contamination in the plastic hypodermic syringes. However, the results for the most part were reliable and proved useful.

The oxygen isotope composition of water was determined by measuring the $\delta^{18}O$ value of CO_2 which had equilibrated with a given water sample overnight at 25°C (Epstein and Mayeda, 1953).

Oxygen isotope analyses of the CO_2 were conducted on a stable isotope mass spectrometer constructed with basic Micromass 903 components.

Materials and methods specific to a given series of experiments are described within the text of that series.

R SERIES EXPERIMENTS: Oxidation of Pentlandite using different additions of $^{18}O_2$.

The T. ferrooxidans cultures were incubated in duplicate in 250 mL Erlenmeyer flasks which were sealed with rubber septa, and contained the following: 1.5 g pentlandite, 45 mL autotrophic media and 5 mL of inoculum. The following volumes of $^{18}O_2$ were injected at atmospheric pressure into the headspaces of the Erlenmeyer flasks: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 12 mL. A set of control flasks without the inocula, but containing equivalent volumes of $^{18}O_2$ injected into the headspace were also prepared. All of the flasks were incubated for six days at 25°C on a rotary shaker (150 rpm). At the end of the experiment, oxygen isotope analyses were conducted on SO_4^{2-}, O_2, and H_2O as described previously.

The data are presented in Table I and Figures 1 and 2. It is seen that the $\delta^{18}O$ value of the bacterially produced SO_4^{2-} increased with increased $^{18}O_2$ additions (Table I).

It is important to note that the $\delta^{18}O$ value of the water also increased in a linear fashion with the extent of $^{18}O_2$ addition (Table I, Figure 1). This did not occur in the absence of the bacterial inoculum (Table I, Figure 1).

The basic question is whether the H_2O acquired ^{18}O from O_2 by exchange processes or through production during the reaction. Hence simplified isotopic balance calculations were carried out. The simplifications include neglecting ^{17}O which is only 0.04 percent abundant, using 18g/mole for water although its δ-value had changed, and ignoring

decreases in the amounts of H_2O and O_2 due to incorporation into SO_4^{2-}. These simplifications introduce less error than the actual measurements of very high $\delta^{18}O$ values in the SO_4^{2-}.

Below is a sample calculation for Sample R-21. The first fraction is the conversion of the amount of a given molecule to moles. The next multiplier (0.9976, 0.002, etc) is the abundance of the particular isotope ^{18}O or ^{16}O. Multipliers of 2 and 4 are used for O_2 and SO_4^{2-}, corresponding to the number of O-atoms in the species.

INITIAL:

H_2O $\quad \frac{50\ g}{18 g/mole} \times 0.0020$ or 5.5×10^{-3} moles ^{18}O

$\quad \frac{50\ g}{18 g/mole} \times 0.9976$ or 2.7711 moles ^{16}O

O_2 (added) $\quad \frac{10^{-1}\ l\text{-atm}}{22.4\ l\text{-atm/mole}} \times 0.965 \times 2 \frac{atoms}{molecule}$ or 8.62×10^{-4} moles ^{18}O

$\quad \frac{10^{-1}\ l\text{-atm}}{22.4\ l\text{-atm/mole}} \times 0.035 \times 2$ or 3.13×10^{-5} moles ^{16}O

O_2 (original) $\quad \frac{0.190\ l\text{-atm}}{22.4\ l\text{-atm/mole}} \times 0.0020 \times 2$ or 3.39×10^{-5} moles ^{18}O

$\quad \frac{0.190\ l\text{-atm}}{22.4\ l\text{-atm/mole}} \times 0.9976 \times 2$ or 1.692×10^{-2} moles ^{16}O

H_2O $\frac{50\ g}{18 g/mole}$ x 0.0022 or 6.11×10^{-3} mole ^{18}O

$\frac{50\ g}{18 g/mole}$ x 0.9974 or 2.7706 moles ^{16}O

O_2 $\frac{0.2\ l\text{-atm}}{22.4\ l\text{-atm/mole}}$ x 0.020 x 2 = 3.57×10^{-4} moles ^{18}O

$\frac{0.2\ l\text{-atm}}{22.4\ l\text{-atm/mole}}$ x 0.9976 x 2 = 1.781×10^{-2} moles ^{16}O

SO_4^{2-} Note that a $\delta^{18}O$ value of 8713 corresponds to an $^{18}O/^{16}O$ ratio of $^{18}O/^{16}O = \frac{0.002}{0.9976}\left[\frac{8713 + 1}{1000}\right] = 0.0175$

3.0×10^{-4} moles x 4 x 0.0175 = 2.1×10^{-5} moles ^{18}O

3.0×10^{-4} moles x 4 x 0.972 = 1.16×10^{-3} moles ^{16}O

The calculations for sample R-21 are summarized in Table II. Interestingly, the larger errors occur with the more abundant ^{16}O because 6 significant figures are required to specify the amount of atoms which have participated. The important conclusion to be reached from these approximate calculations is that the number of ^{18}O atoms which exchanged between O_2 and H_2O is 25 times that which was incorporated into SO_4^{2-}.

It is also seen that the $\delta^{18}O$ value of O_2 at the end of the experiment fell in the range +16 to +23 °/₀₀ with no clear dependence upon its initial $\delta^{18}O$ value. Since atmospheric O_2 has a $\delta^{18}O$ value near + 23 °/₀₀ (Horibe et al., 1973), atmospheric contamination would only realize $\delta^{18}O$ values lower than +23 °/₀₀ in the cases of samples R-1 and R-2. Therefore, the data reinforce the interpretation that oxygen atoms were exchanged between O_2 and H_2O.

TABLE 1

R SERIES - BACTERIAL PENTLANDITE OXIDATION EXPERIMENTS

(Distilled Ottawa tap water with varying $^{18}O_2$ additions in sealed flasks)

Sample No.	$^{18}O_2$ Added (ml)	$\delta^{18}O$ O_2 ($\times 10^4$)	SO_4^{2-} produced mmoles S/l	$\delta^{18}O$ Bacterial $BaSO_4$	Bacterial H_2O	Bacterial O_2	Control	Control H_2O
R-1	0.00	0.0023	5.1	-6.2	-6.2	18.0	R-25	-6.54
R-2			4.3	9.6	-8.9	21.8		
R-3	1.00	0.92	4.6	1499	4.3	22.0	R-26	-10.03
R-4			4.8	1499	5.9	20.4		
R-5	2.00	1.83	4.7	2506	13.8	21.5	R-27	-10.31
R-6			5.8	2054	14.1	16.4		
R-7	3.00	2.75	2.6	3921	25.6	19.9	R-28	-9.45
R-8			5.1	3941	27.6	21.0		
R-9	4.00	3.67	6.0	3847	39.0	18.1	R-29	-9.96
R-10			4.9	5614	39.5	18.4		
R-11	5.00	4.58	4.8	6654	49.3	24.1	R-30	-10.14
R-12			5.6	5426	47.9	17.0		
R-13	6.00	5.50	5.2	6022	54.7	21.9	R-31	-9.63
R-14			4.8	7121	52.2	23.2		
R-15	7.00	6.42	4.6	8345	66.7	18.9	R-32	-8.79
R-16			5.1	8254	70.0	22.7		
R-17	8.00	7.33	4.9	8731	80.3	19.9	R-33	-9.66
R-18			5.4	8735	79.0	18.2		
R-19	9.00	8.25	5.1	9102	77.8	20.5	R-34	-9.56
R-20			5.8	6211	69.4	23.3		
R-21	10.00	9.17	6.0	8713	97.5	17.0	R-35	-9.51
R-22			5.9	8951	105.5	22.6		
R-23	12.00	11.00	5.1	12628	123.0	17.1	R-36	-8.38
R-24			5.6	12311	119.9	21.1		

TABLE II Summary of Isotopic Balance Calculations for Sample R-21

		Moles ^{16}O	Moles ^{18}O
H_2O	INITIAL	2.7711	5.55×10^{-3}
	FINAL	2.7706	6.11×10^{-3}
	Δ	-5×10^{-4}	$+5.6 \times 10^{-4}$
O_2	INITIAL	1.692×10^{-2}	8.96×10^{-4}
	FINAL	1.781×10^{-2}	3.57×10^{-4}
	Δ	8.9×10^{-4}	-5.4×10^{-4}
SO_4^{2-}	INITIAL	0	0
	FINAL	1.16×10^{-3}	2.1×10^{-5}
	Δ	$+1.16 \times 10^{-3}$	$+2.1 \times 10^{-5}$

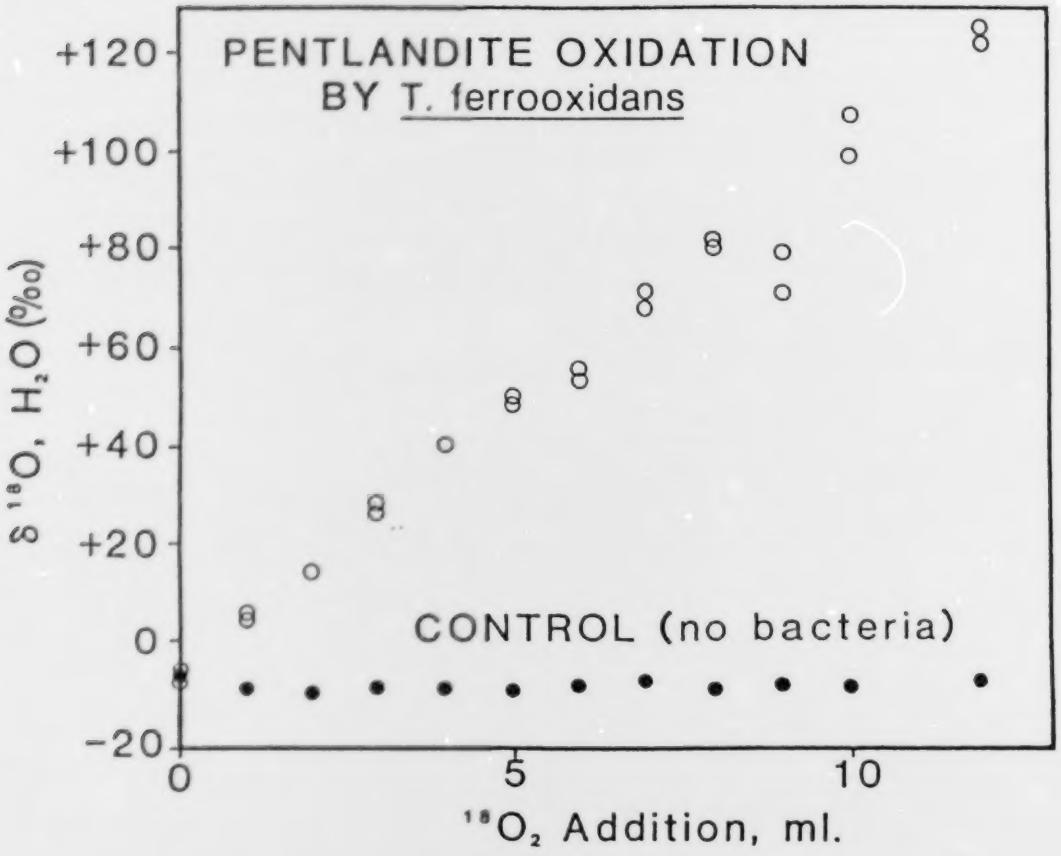

Figure 1 Dependence of the $\delta^{18}O$ value of H_2O on amount of added $^{18}O_2$ during pentlandite oxidation by T. ferrooxidans.

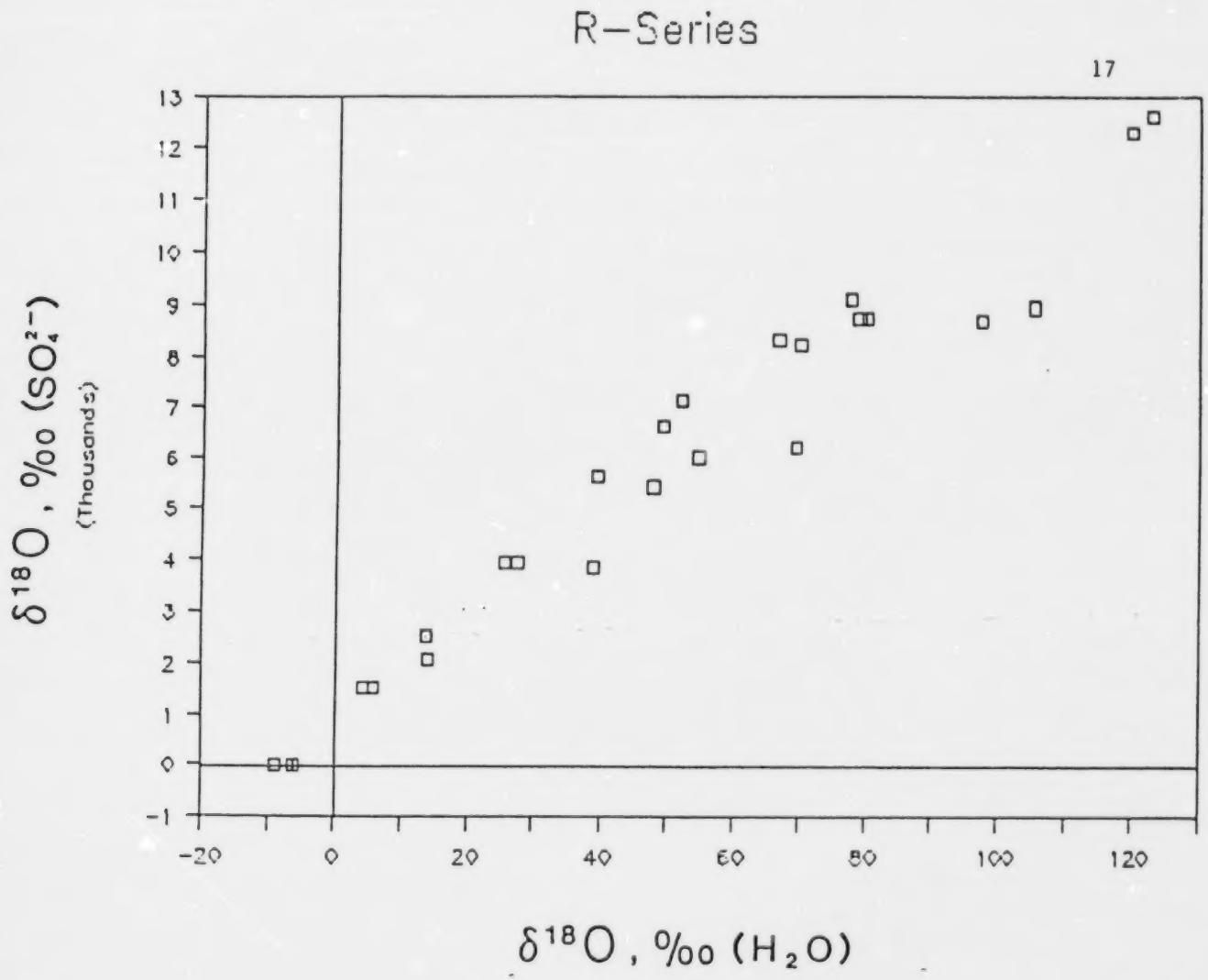

Figure 2 Dependence of $\delta^{18}O$ value of SO_4^{2-} upon that of H_2O during pentlandite oxidation by T. ferrooxidans. Water was initially distilled Ottawa tap water for all experiments but varied amounts of $^{18}O_2$ were added to the sealed reaction flasks.

S-SERIES EXPERIMENTS: Oxidation of pentlandite and sphalerite in sealed flasks with different $\delta^{18}O$ values of H_2O

The S-series of experiments consisted of 6 days incubation of pentlandite (S-1 to S-12) and sphalerite (S-13 to S-24) in sealed containers with waters of different $\delta^{18}O$ values.

The data are presented in Table III and Figure 3. It is noted that the yields from sphalerite were so low that reliable $\delta^{18}O$ determinations on SO_4^{2-} could not be obtained. It is seen that O_2 in these reaction flasks maintained the atmospheric $\delta^{18}O$ value within the measurement errors. This does not rule out exchange since the $\delta^{18}O$ values of the H_2O were not sufficiently different from the O_2 to detect exchange of the same extent noted in the R-series experiment.

The yields with pentlandite were more substantial and the $\delta^{18}O$ value of the SO_4^{2-} increased with that of the water (Figure 3) according to the linear relationship

$$\delta^{18}O\ (SO_4^{2-}) = (0.74 \pm 0.01)\ \delta^{18}O\ (H_2O) + (9.0 \pm 3.3) \quad r^2=1.0$$

As with sphalerite, the $\delta^{18}O$ value of the O_2 was near the atmospheric value but oxygen isotope exchange with water is not ruled out.

TABLE III

S SERIES - BACTERIAL OXIDATION OF PENTLANDITE AND SPHALERITE

(6 days incubation in sealed flasks containing air and waters with different $\delta^{18}O$ values)

$H_2{}^{18}O$ added	CONTROL $\delta^{18}O$		PENTLANDITE				SPHALERITE		
				$\delta^{18}O$				$\delta^{18}O$	
	H_2O	O_2	SO_4^{2-} Produced m moles S/l	H_2O	SO_4^{2-}	O_2	SO_4^{2-} Produced m moles S/l	H_2O	O_2
0			4.1	-10.1	+3.0	+22.9	0.2	-8.3	
0			4.3	-5.5	+4.2	+22.8	0.4	-5.7	
.008	*		4.3	*	+32.8	+22.4	0.4	+34.7	+22.5
.008			3.9	+35.5	+34.5	+22.3	0.1	+34.2	+23.0
.016	+81.4	+22.0	3.6	+78.3	+63.4	+20.9	0.4	+77.6	+22.6
.016			4.0	+81.5	+70.2	+22.9	0.3	+77.4	+22.8
.024	+125.1	+22.4	4.5	+122.4	+94.1	+22.5	0.4	+124.9	+23.3
.024			3.8	+123.5	+103.4	+21.1	0.3	+122.3	+20.9
.032			3.6	+165.3	+133.2	+22.2	0.3	+161.7	+22.9
.032			3.8	+162.7	+133.9	+20.7	0.3	+165.0	+22.8
.040			3.3	+204.9	+157.0	+21.6	0.3		+23.0
.040			3.2	+208.0	+159.9	+21.0	0.2	+208.0	

* Bottle broken in shipping

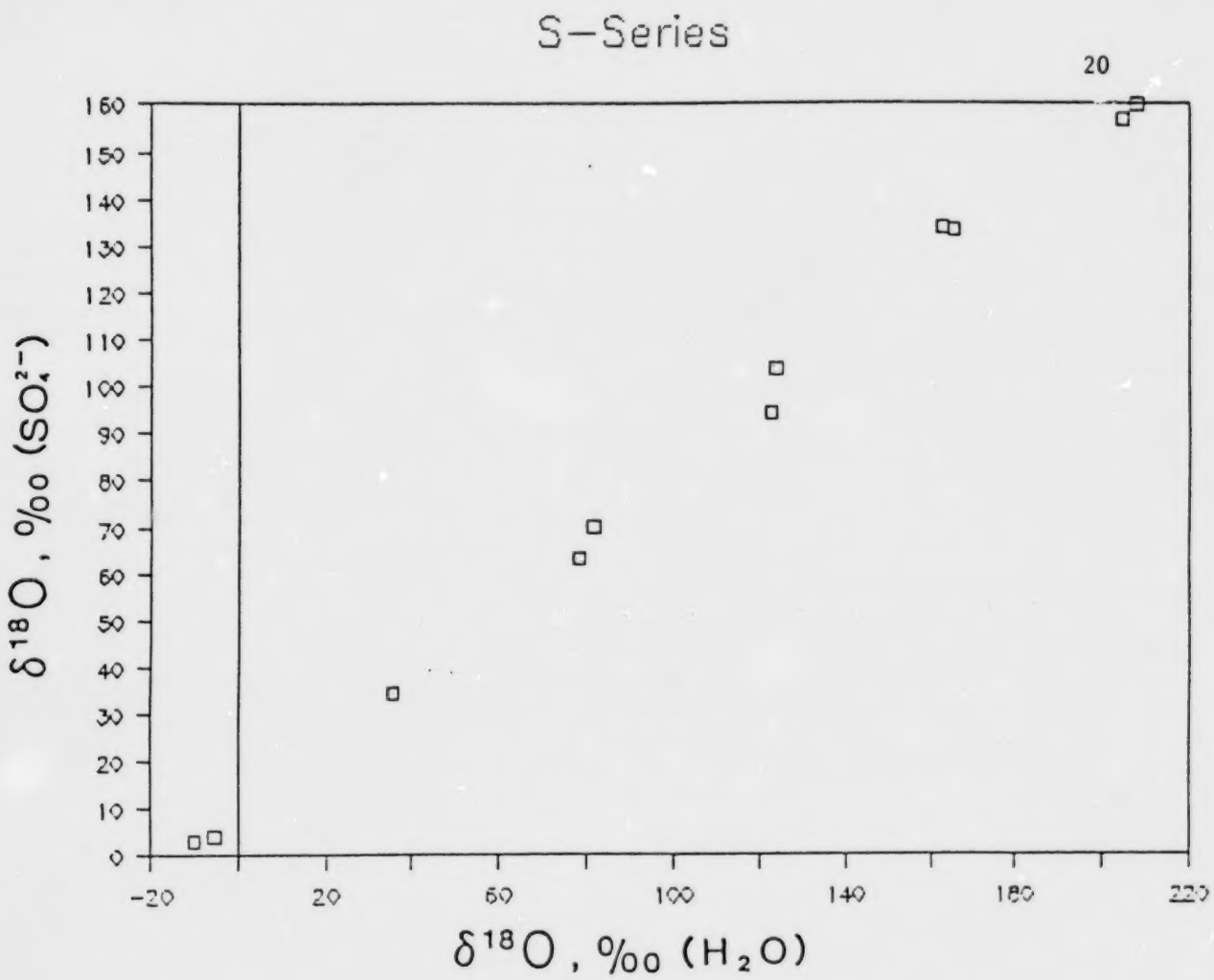

Figure 3 $\delta^{18}O$ values of SO_4^{2-} versus those of H_2O for pentlandite oxidation by T. thiooxidans (6 days incubation in waters of different $\delta^{18}O$ values).

T-SERIES EXPERIMENTS: Oxidation of Pyrite

In these experiments, 10 g of pyrite were reacted with 900 ml of medium plus 100 ml of inoculum in open Fernbach flasks. Sampling of SO_4^{2-} was carried out weekly up to 5 weeks.

The data are shown in Table IV and Figures 4 to 8.

It is seen that the $\delta^{18}O$ of the SO_4^{2-} is related to that of the water by a linear relationship.

$$\delta^{18}O(SO_4^{2-}) = m\ \delta^{18}O(H_2O) + b$$

with the following values for the slope "m" and intercept "b"

Reaction Time (days)	m	b	r^2
7	0.85±0.02	0.3±3.7	0.997
14	0.88±0.01	3.0±2.0	0.999
21	0.89±0.02	3.4±4.4	0.997
28	0.83±0.02	2.7±3.9	0.996
35	0.83±0.06	3.4±9.7	0.979

TABLE IV

T-SERIES - ATMOSPHERIC OXIDATION OF PYRITE BY *T. ferrooxidans* WITH DIFFERENT INITIAL $\delta H_2{}^{18}O$ VALUES

Sample No.	Time (Days)	$SO_4{}^{2-}$ (mmol S/l)	ml $H_2{}^{18}O$/L	H_2O	$SO_4{}^{2-}$
Inoculum	0				-4.4
T-1-0	0		0	-10.5	
T-1-1	7	13.8		-9.7	-6.2
T-1-2	14	28.6		-9.5	-3.2
T-1-3	21	49.3		-9.3	-4.0
T-1-4	28	69.0		-8.4	-2.7
T-1-5	35	89.4			-4.8
T-2-0	0		0.5	+15.2	
T-2-1	7	13.5		+15.5	+15.1
T-2-2	14	32.0		+15.8	+16.6
T-2-3	21	51.2		+16.3	+16.4
T-2-4	28	74.1		+17.4	+15.9
T-2-5	35	99.4			+16.7
T-3-0	0		1.0	+41.2	
T-3-1	7	6.8		+41.3	+35.1
T-3-2	14	18.6		+41.4	+37.7
T-3-3	21	36.1		+41.6	+38.0
T-3-4	28	59.2		+42.7	+38.3
T-3-5	35	82.9			
T-4-0	0		1.5	+66.7	
T-4-1	7	14.8		+67.4	+57.0
T-4-2	14	31.7		+66.6	+60.3
T-4-3	21	55.4		+67.1	+63.1
T-4-4	28	71.6		+67.8	+54.3
T-4-5	35	90.4			
T-5-0	0		2.0	+98.6	
T-5-1	7	17.3		+97.2	+81.8
T-5-2	14	35.8		+97.7	+88.9
T-5-3	21	61.8		+97.2	+91.3
T-5-4	28	80.4		+97.6	+84.0
T-5-5	35	105.1			+90.7

TABLE IV Continued

T-SERIES - ATMOSPHERIC OXIDATION OF PYRITE BY T. ferrooxidans WITH DIFFERENT INITIAL $\delta H_2{}^{18}O$ VALUES

Sample No.	Time (Days)	SO_4^{2-} (mmol S/l)	ml $H_2{}^{18}O$/L	H_2O	SO_4^{2-}
T-6-0	0		2.5	+120.1	
T-6-1	7	12.5		+118.1	+98.5
T-6-2	14	27.8		+119.3	+106.3
T-6-3	21	59.9		+119.0	+108.0
T-6-4	28	75.8		+119.6	+106.4
T-6-5	35	96.4			+97.9
T-7-0	0		3.0	+147.7	
T-7-1	7	10.4		+146.5	+126.4
T-7-2	14	27.4		+146.7	+135.1
T-7-3	21	59.5		+146.6	+126.4
T-7-4	28	75.3		+147.1	
T-7-5	35	93.9			+139.4
T-8-0	0		3.5	+166.7	
T-8-1	7	12.5		+163.9	+138.8
T-8-2	14	30.6		+164.1	+146.6
T-8-3	21	61.2		+164.3	+155.8
T-8-4	28	74.5		+164.8	+147.0
T-8-5	35	88.3			+129.3
T-9-0	0		4.0	+206.4	
T-9-1	7	13.4			+183.3
T-9-2	14	31.5		+205.2	+166.3
T-9-3	21	63.8		+205.0	+186.8
T-9-4	28	78.4		+247.9	+168.9
T-9-5	35	95.4			

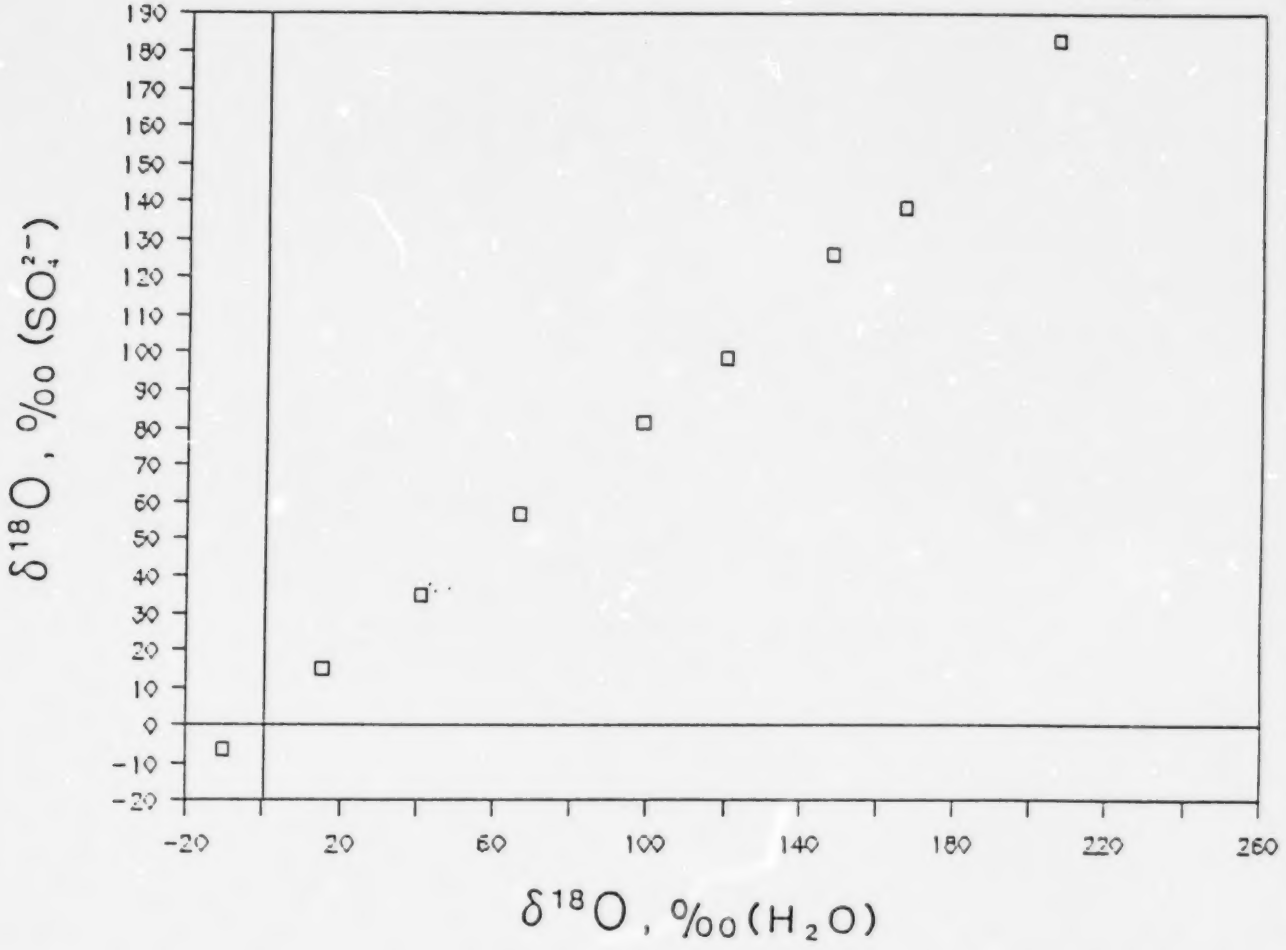

Figure 4 Dependence of $\delta^{18}O$ value of SO_4^{2-} on that of water during atmospheric oxidation of T. ferrooxidans (EL-5). Reaction time, 1 week.

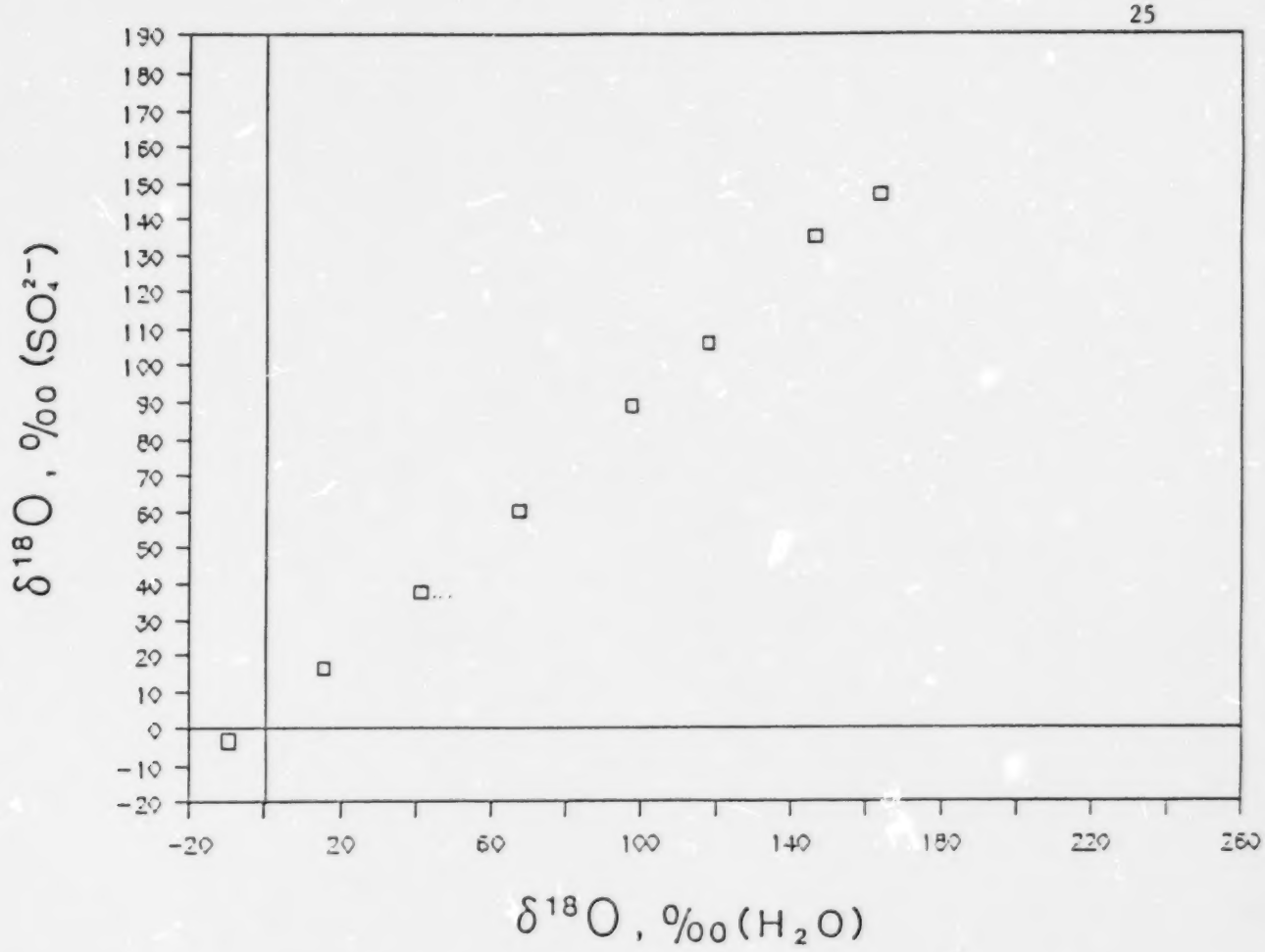

Figure 5 Dependence of $\delta^{18}O$ value of SO_4^{2-} on that of water during atmospheric oxidation of T. ferrooxidans (EL-5). Reaction time, 2 weeks.

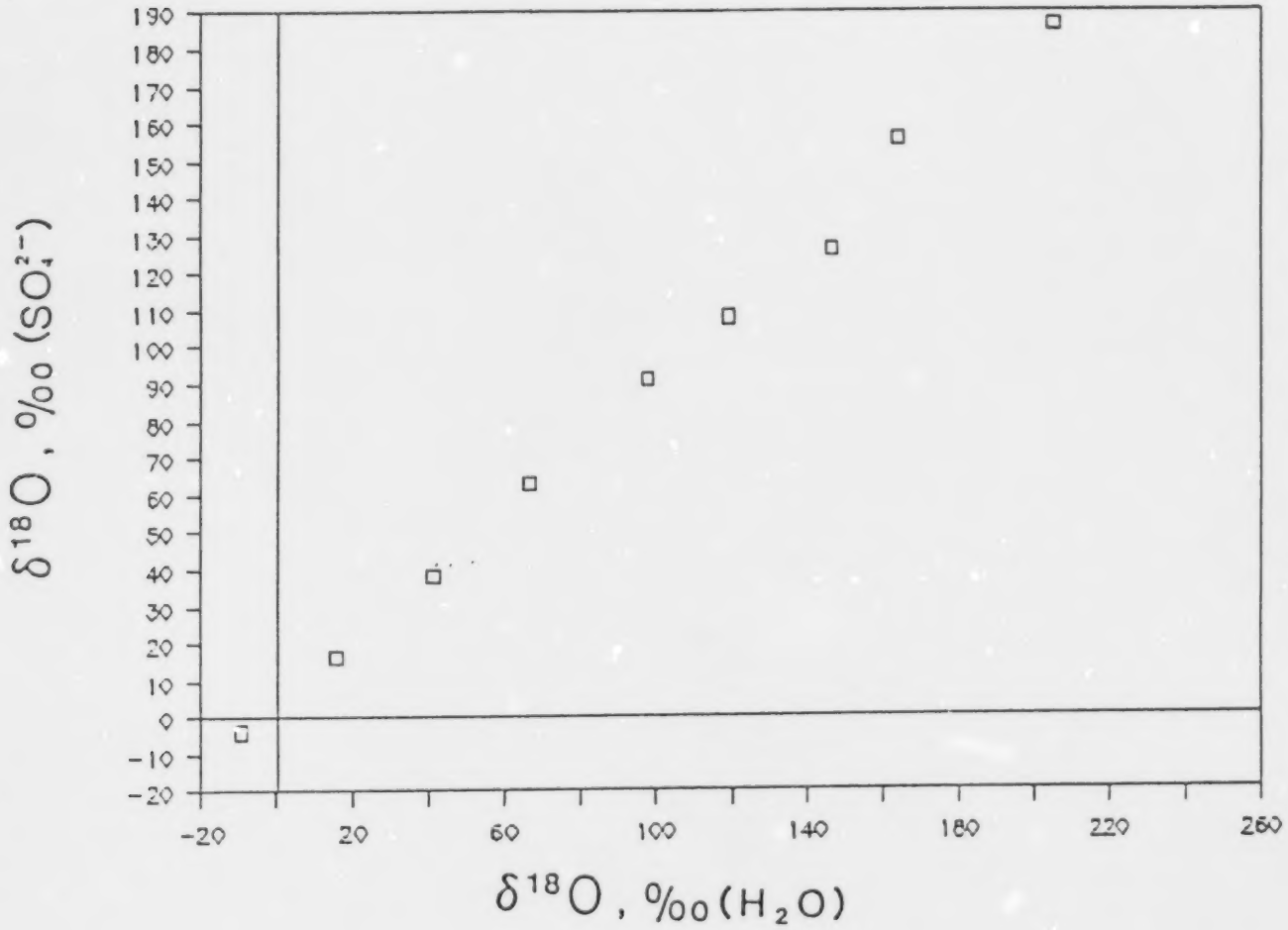

Figure 6 Dependence of $\delta^{18}O$ value of SO_4^{2-} on that of water during atmospheric oxidation of T. ferrooxidans (EL-5). Reaction time, 3 weeks.

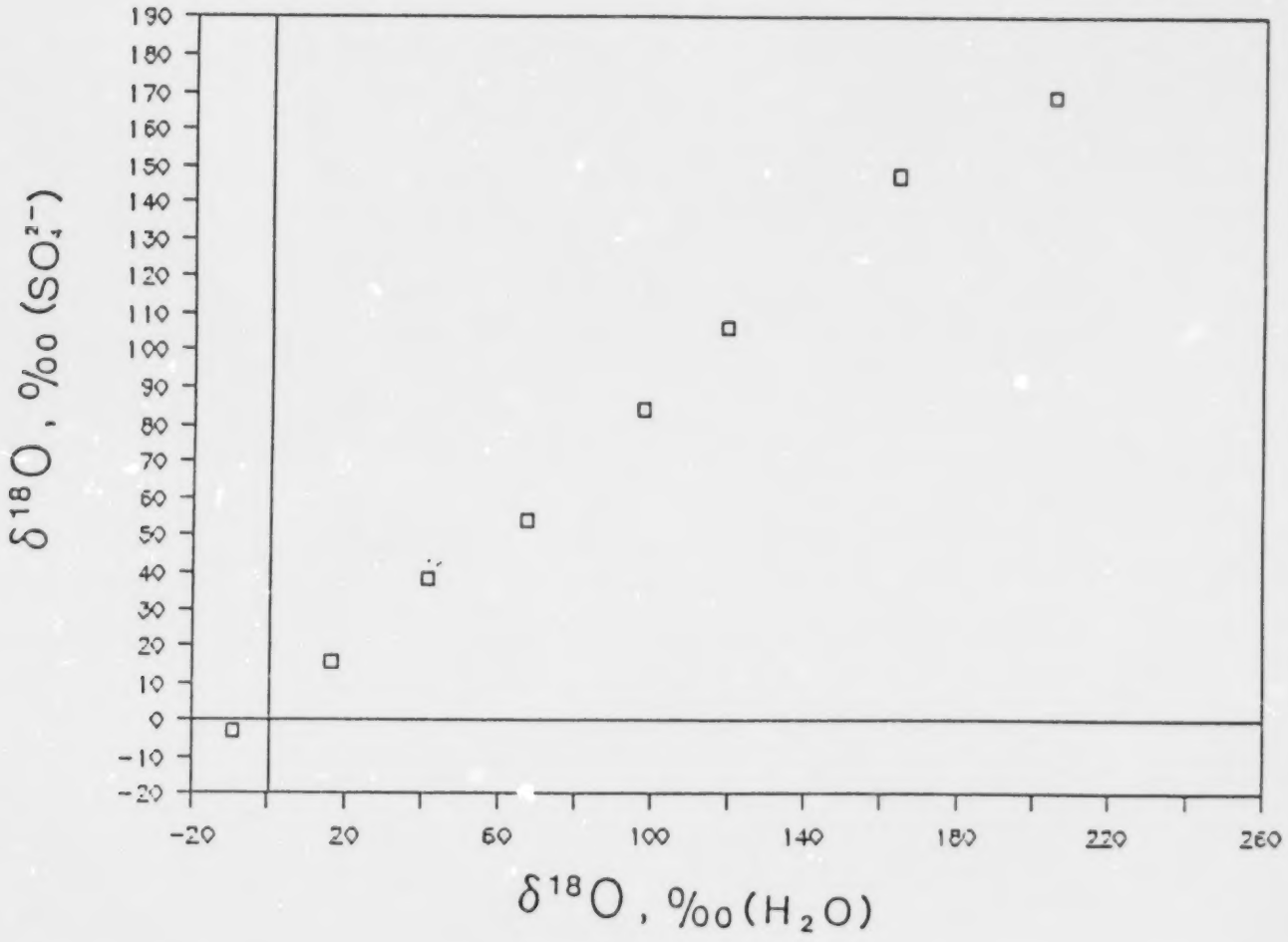

Figure 7 Dependence of $\delta^{18}O$ value of SO_4^{2-} on that of water during atmospheric oxidation of T. ferroxidans (EL-5). Reaction time, 4 weeks.

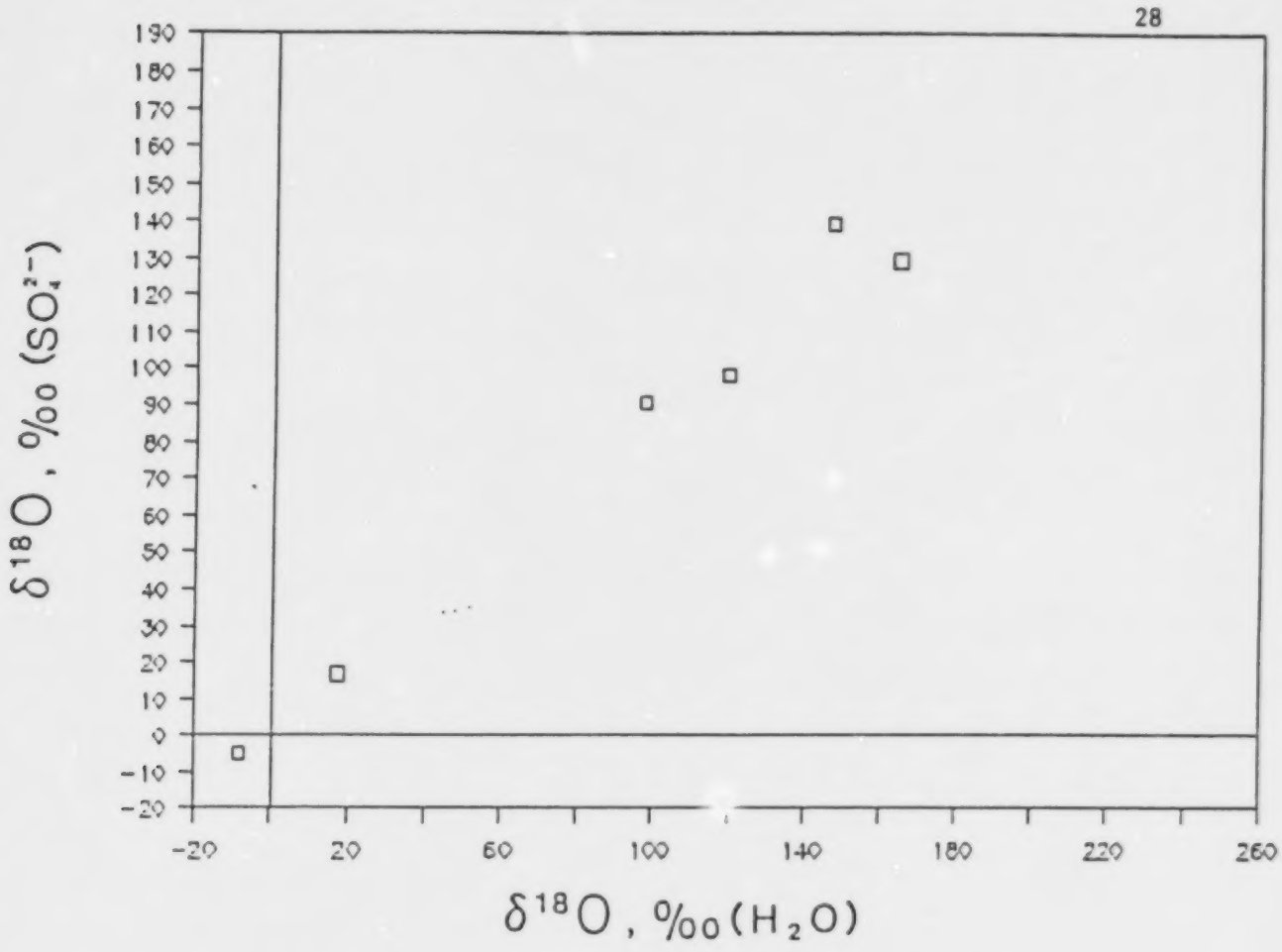

Figure 8 Dependence of $\delta^{18}O$ value of SO_4^{2-} on that of water during atmospheric oxidation of T. ferrooxidans (EL-5). Reaction time, 5 weeks.

U-SERIES: Oxidation of pyrite in sealed flasks with different O_2 pressures and water of different $\delta^{18}O$ values.

This experiment was designed to illustrate whether O_2 pressure altered the oxygen isotope composition of SO_4^{2-} produced during bacterial sulphide oxidation.

Erlenmeyer flasks (250 ml) containing 0.5 g pyrite, 45 ml of media, and 5 ml of inoculum, were sealed with rubber stoppers. Additions of $H_2{}^{18}O$ from 0 to 0.08 ml were used as well as O_2 (atmospheric isotope composition) excesses from 0 to 20 ml-atm.

One set of experiments which were carried out in pure O_2 had 0.04 ml of a 1 percent $NaHCO_3$ solution added to provide a carbon source.

Six experiments were conducted for one week and two for two weeks.

The data are summarized in Tables V to XII and Figures 9 to 16.

These experiments had a basic problem in that the inoculum carry over was comparable to the amount of SO_4^{2-} subsequently produced. Therefore corrections were large and slight loss of $BaSO_4$ or variations in the mass of a filter disk could realize large errors in the corrected $\delta^{18}O$ value. In Tables V to XII, corrections are made two ways. Method 1, used the actual weight of $BaSO_4$. Method 2 used an average of the three largest consistent yields. In the second method, losses of $BaSO_4$ could be compensated.

An unknown is the concentrations of reaction intermediates transferred with the inoculum. This could be obtained with the help of metal ion concentrations, provided that no metallic minerals precipitated. A better method, as proposed in the previous report, would be to grow the inoculum on sulphide which differed significantly in sulphur isotope composition from the minerals used in the experiments and use sulphur isotope data for corrections.

There is a further problem even when the mass of transferred intermediates is known. One does not know how much of the oxygen in their product sulphate was determined by the inoculum and how much by the reaction conditions. However, calculations can be made assuming either extreme and compared to the measured data. This comparison per se might prove informative in deciphering the mechanism.

In light of the inoculum carry over problem, discussed above, one must consider the data of this experiment cautiously. It is seen in Tables VII to IX, that there is a slight increase in SO_4^{2-} formed with increasing O_2 pressure (Table XIII). In the case of pure O_2, however, the yields are the lowest. This may relate to the different carbon source. The yields at the end of two weeks are roughly double those of one week incubations and for the two cases examined, the yield was higher at higher O_2 pressure.

When $\delta^{18}O$ of SO_4^{2-} is plotted against that of H_2O, there are highly correlated linear relationships (Table XII). A number of observations stand out. For the one week

incubations, the slopes are close to unity, the highest found so far in experiments with O_2 of atmospheric $\delta^{18}O$ value. In going from 0 to 15 ml-atm excess O_2, the slope decreases from 1.22 to 0.95, which is consistent with less dependence on the isotopic composition of the water and in turn more dependence of that of O_2 as might be expected. However, at 20 ml-atm excess O_2, the slope has increased slightly although the standard deviation just overlaps with that of the 15 ml-atm excess O_2 run. For the pure O_2 run, U-100-1, the slope is higher at 1.19. This might be explained by the different carbon source.

In contrast, for the two week incubations, the slopes are very low around 0.5 and the slope for 15 ml-atm excess O_2, 0.49, is lower than for 5 ml-atm excess O_2, 0.55.

Thus there is some evidence that more oxygen in the SO_4^{2-} was derived from O_2 with higher initial partial pressures, but the effect is quite small. This will be further discussed later.

Since yields of SO_4^{2-} varied and the inoculum carry over was high, another approach used was to examine how the uncorrected $\delta^{18}O$ value of the SO_4^{2-} related to the SO_4^{2-}yield of water of a given $\delta^{18}O$ value. The plots are given in Figures 17 to 25. In each case, the two points on the right in the plots correspond to the 2 week runs. On the x-axis, the amount of inoculum is subtracted out. This is a constant and does not affect the plots.

Keeping in mind that for increasing SO_4^{2-} production,

the inoculum correction is relatively less, the results are very revealing. There are definite trends. For no $H_2{}^{18}O$ water added, $\delta^{18}O$ of $SO_4{}^{2-}$ increases with $SO_4{}^{2-}$ production (Figure 17). The same is true to a less extent for the 0.01 and 0.02 ml $H_2{}^{18}O$ additions. At higher $H_2{}^{18}O$ additions, the $\delta^{18}O$ value of $SO_4{}^{2-}$ decreases with $SO_4{}^{2-}$ yield, the decrease being greatest for the highest $H_2{}^{18}O$ addition (Figure 25).

There are at least four important points to note. (1) For no $H_2{}^{18}O$ addition, the $\delta^{18}O$ value of the H_2O is similar to that of the inoculum. Yet the $\delta^{18}O$ value of $SO_4{}^{2-}$ increased with its yield. (2) In previous experiments open to the atmosphere, the $\delta^{18}O$ value of the $SO_4{}^{2-}$ changed very little over periods of several weeks (See for example, Table IV in this report). (3) Inoculum carry over has lower $\delta^{18}O$ values in $SO_4{}^{2-}$ than that produced in the experiments with $H_2{}^{18}O$ additions. This mass transfer effect should decrease proportionately with higher $SO_4{}^{2-}$ yields. Yet the measured raw $\delta^{18}O$ value decreased with highest yields in Figures 19 to 25. (4) The basic difference between the U-series of experiments and previous experiments is that the former are closed.

The extremes of slopes for the $\delta^{18}O$ ($SO_4{}^{2-}$) versus $\delta^{18}O$ (H_2O) plots and the large dependence of $\delta^{18}O$ ($SO_4{}^{2-}$) upon $SO_4{}^{2-}$ yields must reflect the closed vessel conditions and limited O_2 supply. For air alone in the flask, there are about 5.7×10^{-2} g of oxygen. In the two week experiment, about 4×10^{-2} g of oxygen have been incorporated into $SO_4{}^{2-}$

from both H_2O and O_2. Dependent upon the fraction derived from each source, a significant amount of O_2 has been depleted. In other words, O_2 pressures of even less than the partial atmospheric pressure must have existed for different fractions of time in many of the experiments.

One would expect that in the two week experiment, the isotopic composition of the SO_4^{2-} should become more dependent upon water as O_2 is consumed. However, this is not consistent with the slope of the $\delta^{18}O$ (SO_4^{2-}) versus $\delta^{18}O$ (H_2O) plot decreasing to 0.5. This implies that there are significant kinetic isotope effects associated with the incorporation of O_2 and possibly other steps in the mechanism. It might also indicate changes in rates of individual steps in the mechanism as the O_2 pressure decreases.

In summary, the U-series of experiments produced thought provoking data. There are definite trends which are much larger than any errors identified with the inoculum background. These in part must relate to kinetic isotope effects and a limited O_2 reservoir. With suitable experiments, these effects can be sorted out. In particular, experiments with different O_2 concentrations should be carried out where the partial pressure of O_2 remains constant. These would provide a base to which data from limited O_2 experiments could be compared.

The U-series of experiments are very relevant to the geochemical interpretation of subsurface sulphide mineral

oxidation. Such systems are likely to have limited O_2 and display the so-called "reservoir effect" i.e. because of kinetic isotope effects, the $\delta^{18}O$ value of unreacted O_2 progressively changes.

Table V U-0-1 Series: Pyrite oxidation by T. ferrooxidans; Ambient O_2; 6 days

Sample	$H_2{}^{18}O$ ml	$BaSO_4$ mg S/ml	$\delta^{18}O$ (^{o}/oo SMOW)			
			$BaSO_4$			
			Measured	1	2	H_2O
Inoculum	0		- 4.1			
U1-0-1	0	0.247	- 6.5	-14.5	-12.5	-10.0
U2-0-1	0.01	0.247	- 3.5	- 1.5	- 2.0	- 0.3
U3-0-1	0.02	0.250	+ 0.6	+15.5	+12.3	+10.6
U4-0-1	0.03	0.267	+ 4.5	+25.8	+25.8	+20.0
U5-0-1	0.04	0.257	+ 7.1	+39.0	+34.9	+30.1
U6-0-1	0.05	0.267	+10.2	+45.6	+45.6	+38.8
U7-0-1	0.06	0.257	+13.0	+75.1	+59.2	+48.6
U8-0-1	0.07	0.247	+16.7	+76.0	+68.3	+56.7
U9-0-1	0.08	0.273	+21.1	+78.6	+83.6	+68.6

Table VI U-5-1 Series: Pyrite oxidation by T. ferrooxidans; 5 ml-atm O_2 excess; 6 days

Sample	$H_2{}^{18}O$ ml	$BaSO_4$ mg S/ml	$\delta^{18}O$ (^{o}/oo SMOW)			
			$BaSO_4$			
			Measured	1	2	H_2O
U1-5-1	0	0.270	- 6.9	-13.5	-13.5	- 9.7
U2-5-1	0.01	0.257	- 3.9	- 3.3	- 3.4	- 0.1
U3-5-1	0.02	0.267	- 0.4	+ 8.8	+ 8.8	+10.7
U4-5-1	0.03	0.267	+ 4.6	+26.2	+26.2	+19.2
U5-5-1	0.04	0.253	+ 7.1	+40.7	+34.9	+29.6
U6-5-1	0.05	0.257	+ 8.1	+42.9	+38.3	+37.5
U7-5-1	0.06	0.257	+12.4	+59.4	+53.3	+47.7
U8-5-1	0.07	0.240	+15.6	+85.9	+61.1	+58.5
U9-5-1	0.08	0.263	+19.9	+82.1	+79.4	+68.6

Table VII U-10-1 Series: Pyrite oxidation by T. ferrooxidans; 10 ml-atm O_2 excess; 6 days

Sample	$H_2{}^{18}O$ ml	$BaSO_4$ mg S/ml	$\delta^{18}O$ (^{o}/oo SMOW)			
			$BaSO_4$			
			Measured	1	2	H_2O
U1-10-1	0	0.257	- 7.6	-17.6	-14.7	- 8.8
U2-10-1	0.01	0.253	- 1.9	+ 4.7	+ 2.6	0.0
U3-10-1	0.02	0.243	+ 2.2	+24.6	+15.0	+10.2
U4-10-1	0.03	0.273	+ 4.7	+24.8	+22.6	+18.5
U5-10-1	0.04	0.270	+ 7.5	+35.1	+31.1	+29.6
U6-10-1	0.05	0.283	+12.4	+46.0	+46.0	+38.7
U7-10-1	0.06	0.297	+15.0	+51.0	+53.9	+48.9
U8-10-1	0.07	0.203	+15.7		+56.0	+56.8
U9-10-1	0.08	0.250	+19.4		+67.2	+67.8

Table VIII U-15-1 Series: Pyrite oxidation by T. ferrooxidans; 15 ml-atm O_2 excess; 6 days

Sample	$H_2{}^{18}O$ ml	$BaSO_4$ mg S/ml	$\delta^{18}O$ (^{o}/oo SMOW)			
			$BaSO_4$			
			Measured	1	2	H_2O
U1-15-1	0	0.313	- 6.7	-10.7	-10.7	- 9.9
U2-15-1	0.01	0.283	- 1.1	- 1.1	+ 3.5	+ 2.7
U3-15-1	0.02	0.323	+ 1.9	+10.5	+10.5	+10.6
U4-15-1	0.03	0.267	+ 3 5	+22.3	+15.2	+19.3
U5-15-1	0.04	0.300	+ 8.9	+31.3	+28.9	+28.6
U6-15-1	0.05	0.307	+15.0	+46.1	+44.4	+38.8
U7-15-1	0.06	0.280	+17.0	+51.4	+49.5	+49.4
U8-15-1	0.07	0.307	+19.5	+69.3	+55.9	+58.5
U9-15-1	0.08	0.293	+20.8	+66.6	+59.2	+68.0

Table IX U-20-1 Series: Pyrite oxidation by T. ferrooxidans; 20 ml-atm O_2; 6 days

Sample	$H_2{}^{18}O$ ml	$BaSO_4$ mg S/ml	$\delta^{18}O$ (°/oo SMOW) $BaSO_4$			
			Measured	1	2	H_2O
U1-20-1	0	0.313	- 7.6	-13.0	-13.0	- 9.6
U2-20-1	0.01	0.283	- 2.9	- 0.5	- 1.1	+ 0.2
U3-20-1	0.02	0.250	+ 0.6	+15.5	+12.3	+10.6
U4-20-1	0.03	0.267	+ 4.0	+24.1	+16.5	+19.2
U5-20-1	0.04	0.300	+ 9.7	+33.5	+31.0	+27.6
U6-20-1	0.05	0.307	+11.5	+36.9	+35.5	+39.1
U7-20-1	0.06	0.280	+14.9	+55.0	+44.2	+49.7
U8-20-1	0.07	0.307	+21.4	+62.9	+60.7	+57.3
U9-20-1	0.08	0.293	+25.4	+79.6	+70.9	+68.3

Table X U-100-1 Series: Pyrite oxidation by T. ferrooxidans; Pure O_2; 0.04 ml of 1% $NaHCO_3$ Solution; 6 days

Sample	$H_2{}^{18}O$ ml	$BaSO_4$ mg S/ml	$\delta^{18}O$ (°/oo SMOW) $BaSO_4$			
			Measured	1	2	H_2O
U1-100-1	0	0.233	- 7.1	-20.3	-20.3	- 9.5
U2-100-1	0.01	0.203	- 3.6	+ 3.5	- 1.4	+ 0.2
U3-100-1	0.02	0.223	- 1.9	+10.6	+ 7.8	+ 9.7
U4-100-1	0.03	0.220	- 0.7	+20.8	+14.2	+18.6
U5-100-1	0.04	0.210	+ 3.7	+77.8	+37.9	+29.4
U6-100-1	0.05	0.210	+ 4.5	+86.2	+42.2	+39.3
U7-100-1	0.06	0.193	+ 5.3	-	+46.5	+49.1
U8-100-1	0.07	0.220	+ 9.4	+94.9	+68.6	+57.4
U9-100-1	0.08	0.203	+ 9.9	-	+71.3	+66.2

Table XI U-5-2 Series: Pyrite oxidation by T. ferrooxidans; 5 ml-atm O_2 excess; 2 weeks

Sample	$H_2{}^{18}O$ ml	$BaSO_4$ mg S/ml	$\delta^{18}O$ (°/oo SMOW)			
			$BaSO_4$			
			Measured	1	2	H_2O
U1-5-2	0	0.460	- 5.1	- 5.8	- 5.7	-10.5
U2-5-2	0.01	0.460	- 1.3	+ 0.8	+ 0.4	- 0.5
U3-5-2	0.02	0.423	+ 4.4	+11.3	+ 9.6	+ 8.3
U4-5-2	0.03	0.493	+ 5.0	+10.4	+10.6	+18.5
U5-5-2	0.04	0.506	+10.4	+19.5	+19.3	+29.1
U6-5-2	0.05	0.413	+13.5	+25.6	+24.3	+38.8
U7-5-2	0.06	0.430	+16.6	+29.0	+29.3	+49.0
U8-5-2	0.07	0.467	+18.8	+36.9	+32.8	+55.4
U9-5-2	0.08	0.513	+21.3	+42.9	+36.9	+67.4

Table XII U-15-2 Series: Pyrite oxidation by T. ferrooxidans; 15 ml-atm O_2 excess; 2 weeks

Sample	$H_2{}^{18}O$ ml	$BaSO_4$ mg S/ml	$\delta^{18}O$ (°/oo SMOW)			
			$BaSO_4$			
			Measured	1	2	H_2O
U1-15-2	0	0.437	- 1.6	+ 0.3	- 0.3	-10.0
U2-15-2	0.01	0.473	- 1.3	+ 0.6	+ 0.1	- 0.1
U3-15-2	0.02	0.487	+ 5.8	+12.1	+10.8	+ 9.8
U4-15-2	0.03	0.503	+ 6.5	+11.9	+ 1.9	+19.0
U5-15-2	0.04	0.453	+10.0	+18.6	+17.1	+29.5
U6-15-2	0.05	0.590	+12.9	+23.2	+21.5	+38.4
U7-15-2	0.06	0.373	+15.2	+29.1	+24.9	+49.5
U8-15-2	0.07	0.503	+18.5	+41.9	+29.9	+54.6
U9-15-2	0.08	0.563	+22.5	+35.1	+35.9	+67.9

TABLE XIII Summary of SO_4^{2-} yields and dependence of $\delta^{18}O$ values of SO_4^{2-} on those of H_2O for U-series experiments

Series	Excess O_2 ml-atm	Average SO_4^{2-} mg S/ml	Slope, m	Intercept, b (°/₀₀)	r^2
One Week Incubation					
U-0-1	0	0.257	1.22±0.02	-0.7±1.1	0.999
U-5-1	5	0.259	1.15±0.05	-1.7±3.4	0.989
U-10-1	10	0.259	1.03±0.06	+1.3±4.2	0.979
U-15-1	15	0.287	0.95±0.05	+0.6±3.9	0.979
U-20-1	20	0.288	1.04±0.05	-1.7±3.5	0.986
U-100-1	Pure O_2	0.213	1.19±0.07	-4.7±5.1	0.977
Two Weeks Incubation					
U-5-2	5	0.463	0.55±0.03	+1.8±1.9	0.986
U-15-2	15	0.487	0.49±0.06	+1.7±4.1	0.918

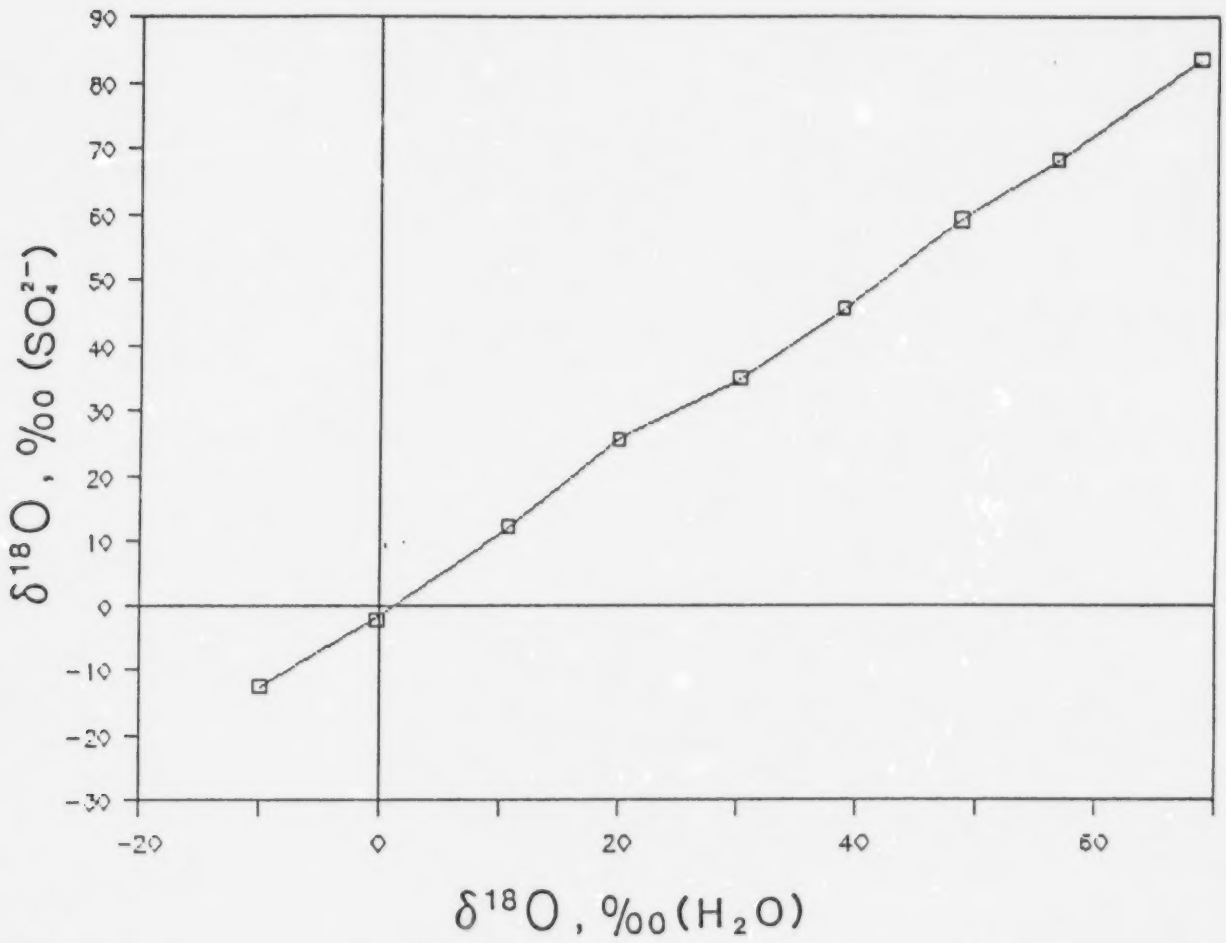

Figure 9 $\delta^{18}O$ of sulphate versus $\delta^{18}O$ of water for pyrite oxidation by T. ferrooxidans (EL-5), ambient O_2, 6 days.

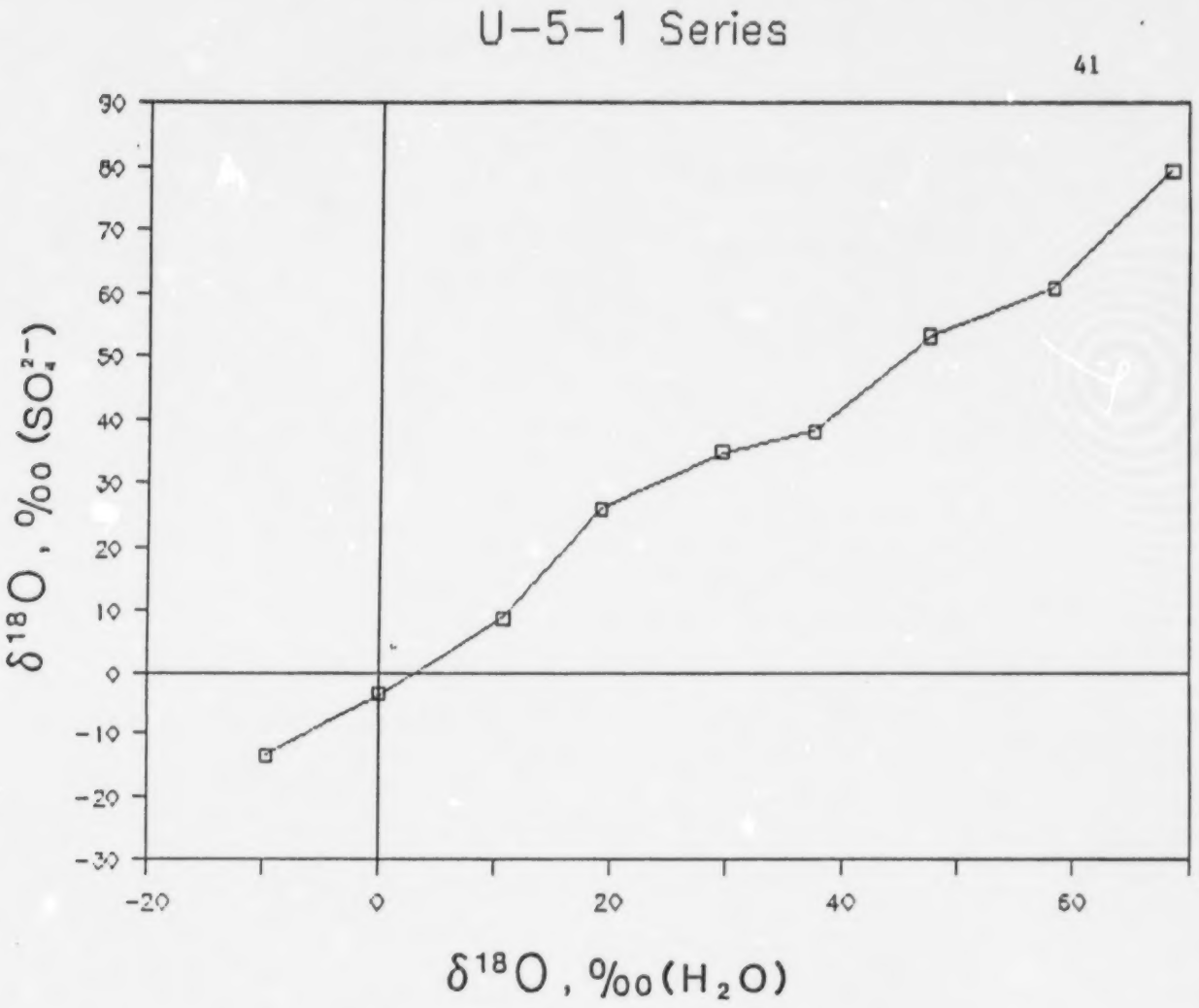

Figure 10 $\delta^{18}O$ of sulphate versus $\delta^{18}O$ of water for pyrite oxidation by T. ferrooxidans (EL-5), 5 ml-atm O_2 excess, 6 days.

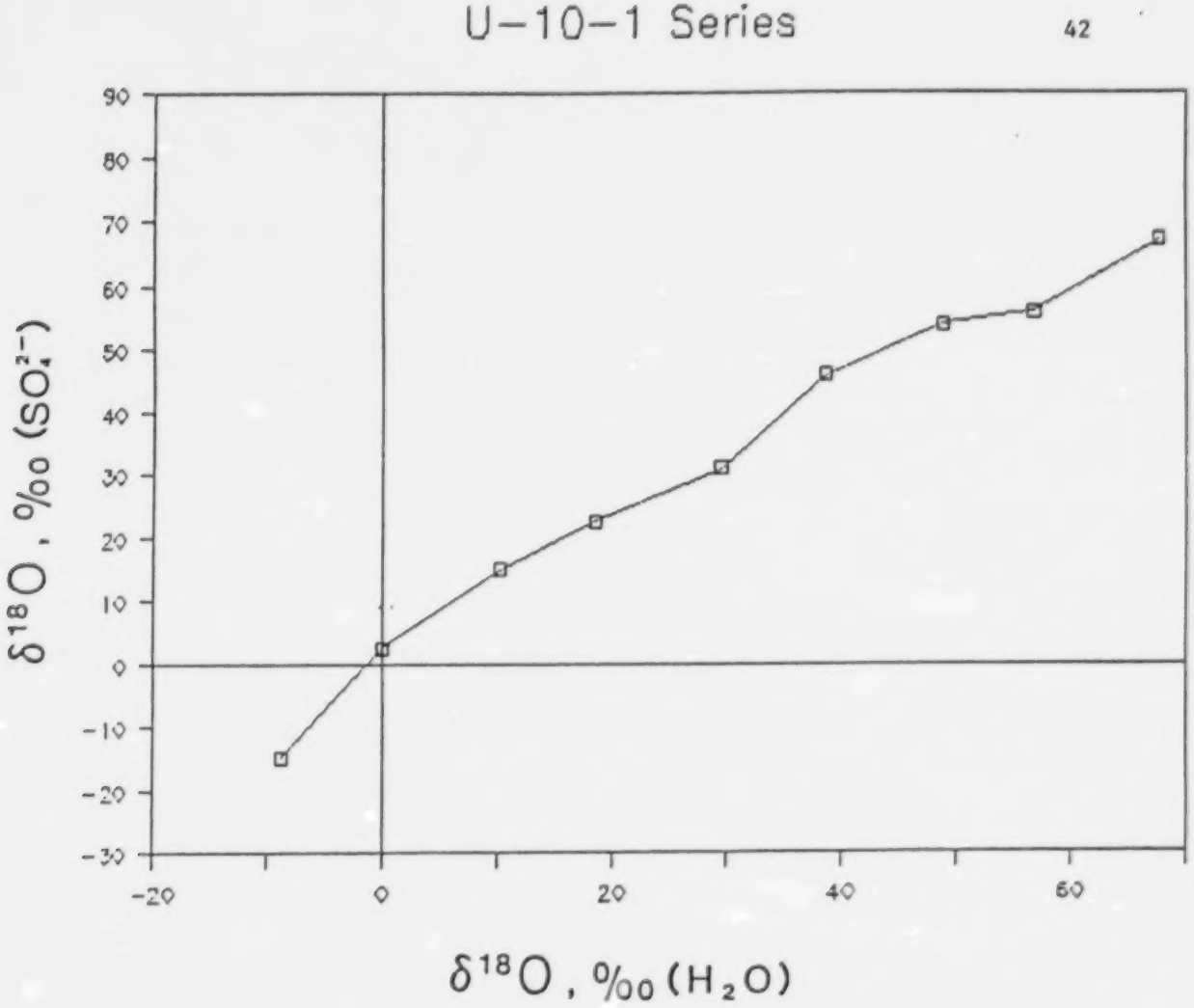

Figure 11 $\delta^{18}O$ of sulphate versus $\delta^{18}O$ of water for pyrite oxidation by T. ferrooxidans (EL-5), 10 ml-atm O_2 excess, 6 days.

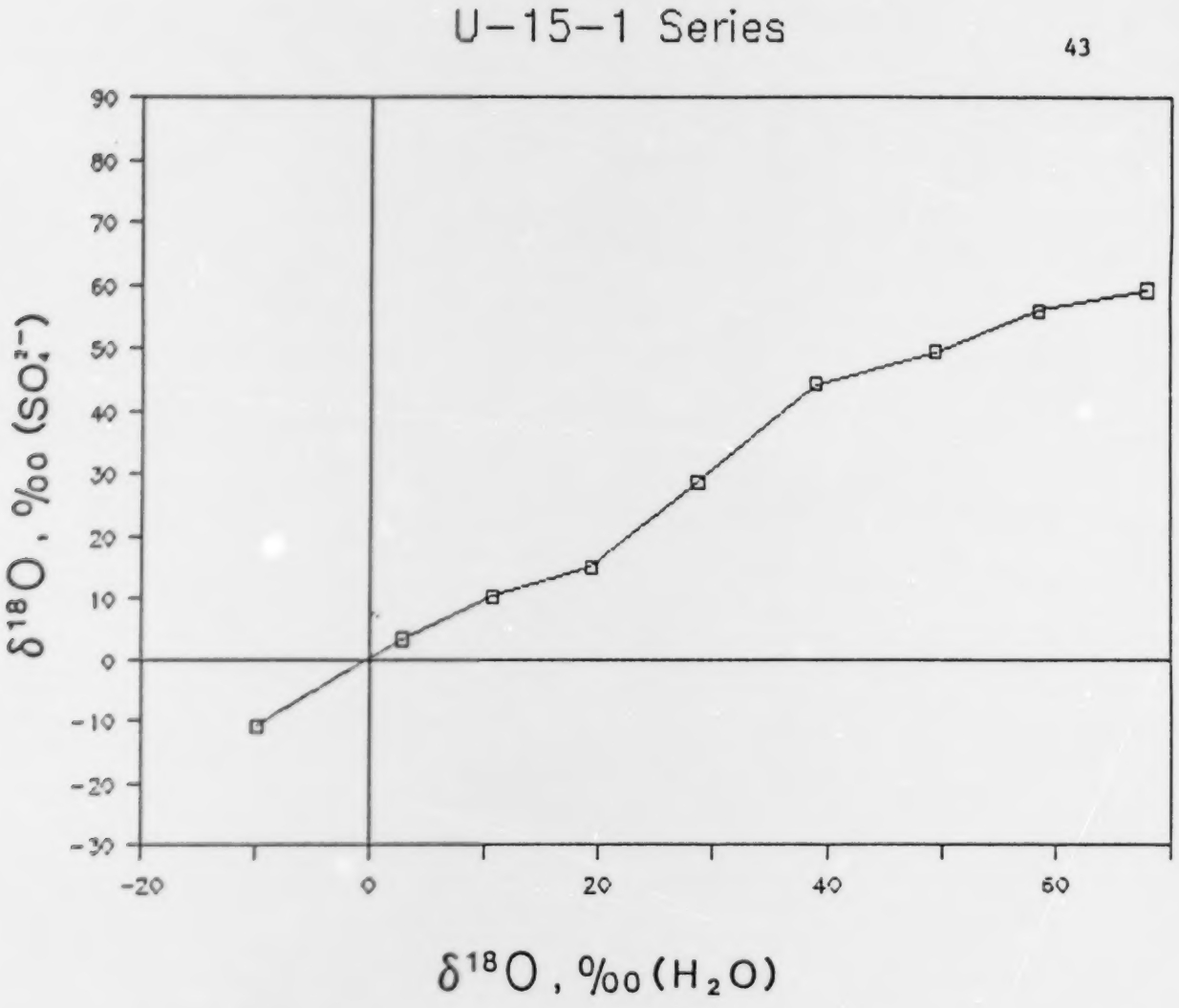

Figure 12 $\delta^{18}O$ of sulphate versus $\delta^{18}O$ of water for pyrite oxidation by T. ferrooxidans (EL-5), 15 ml-atm, O_2 excess, 6 days.

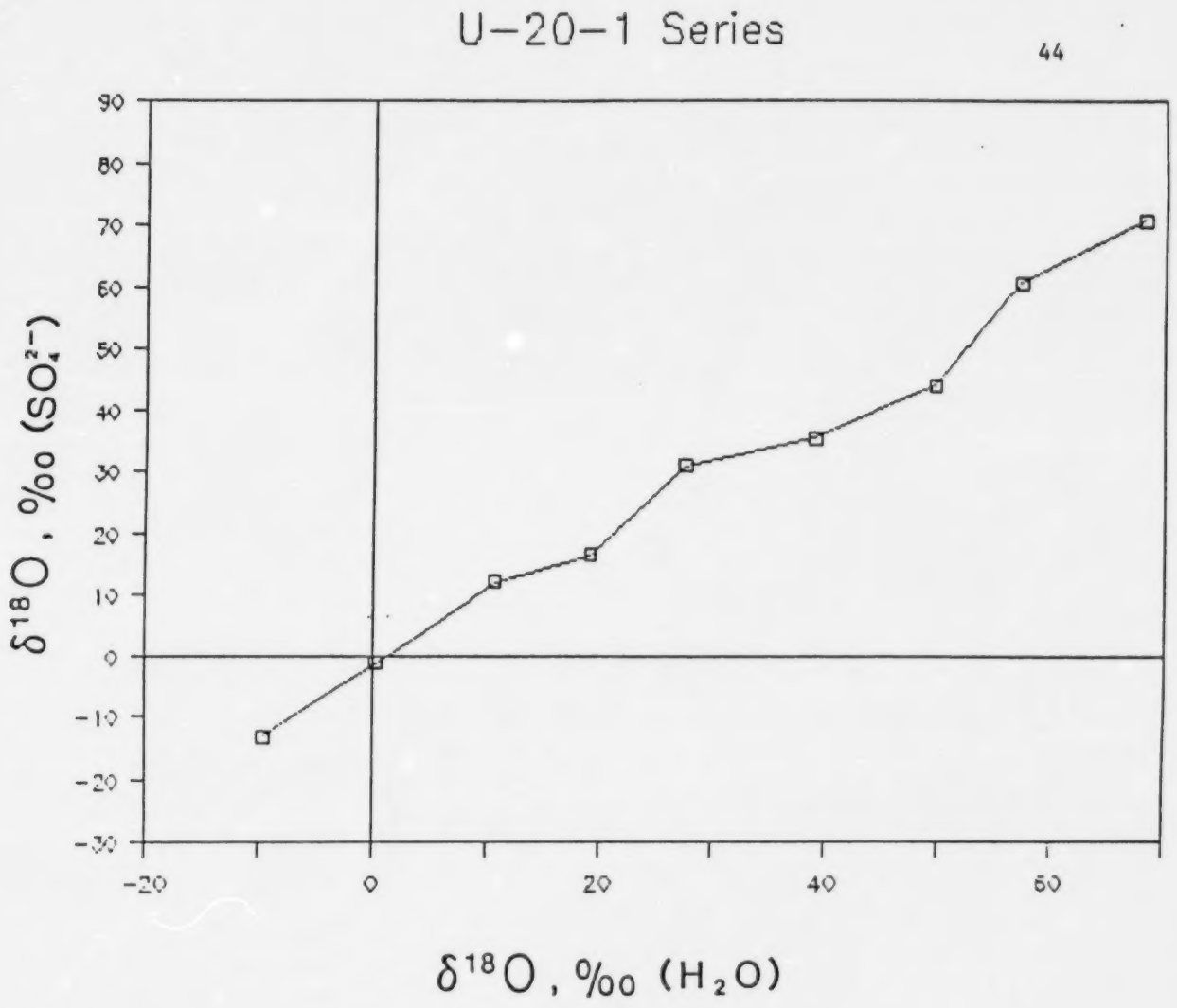

Figure 13 $\delta^{18}O$ of sulphate versus $\delta^{18}O$ of water for pyrite oxidation by T. ferrooxidans (EL-5), 20 ml-atm O_2 excess, 6 days.

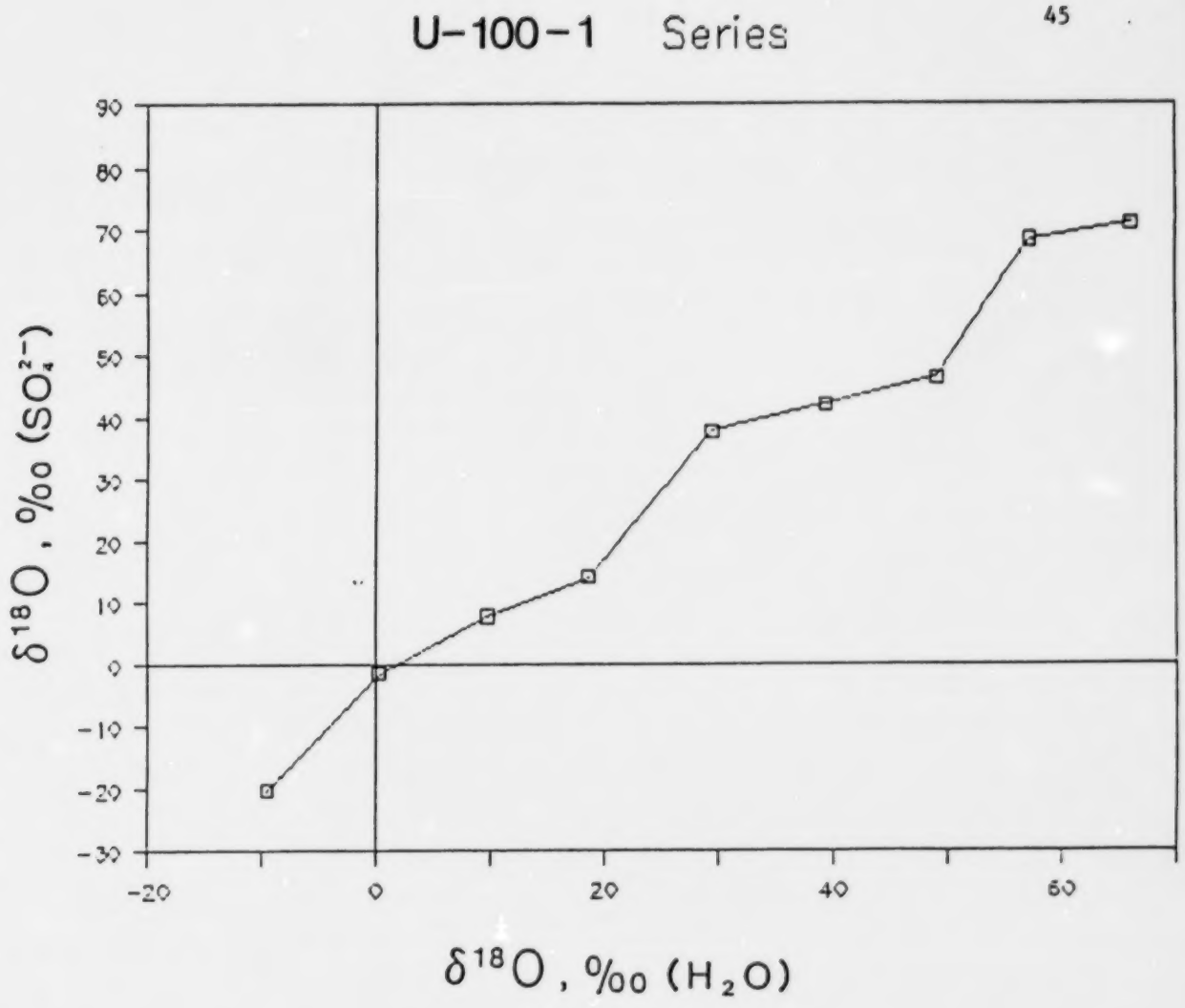

Figure 14 $\delta^{18}O$ of sulphate versus $\delta^{18}O$ of water for pyrite oxidation by T. ferrooxidans (EL-5), pure O_2, 0.04 ml of 1% $NaHCO_3$ solution, 6 days.

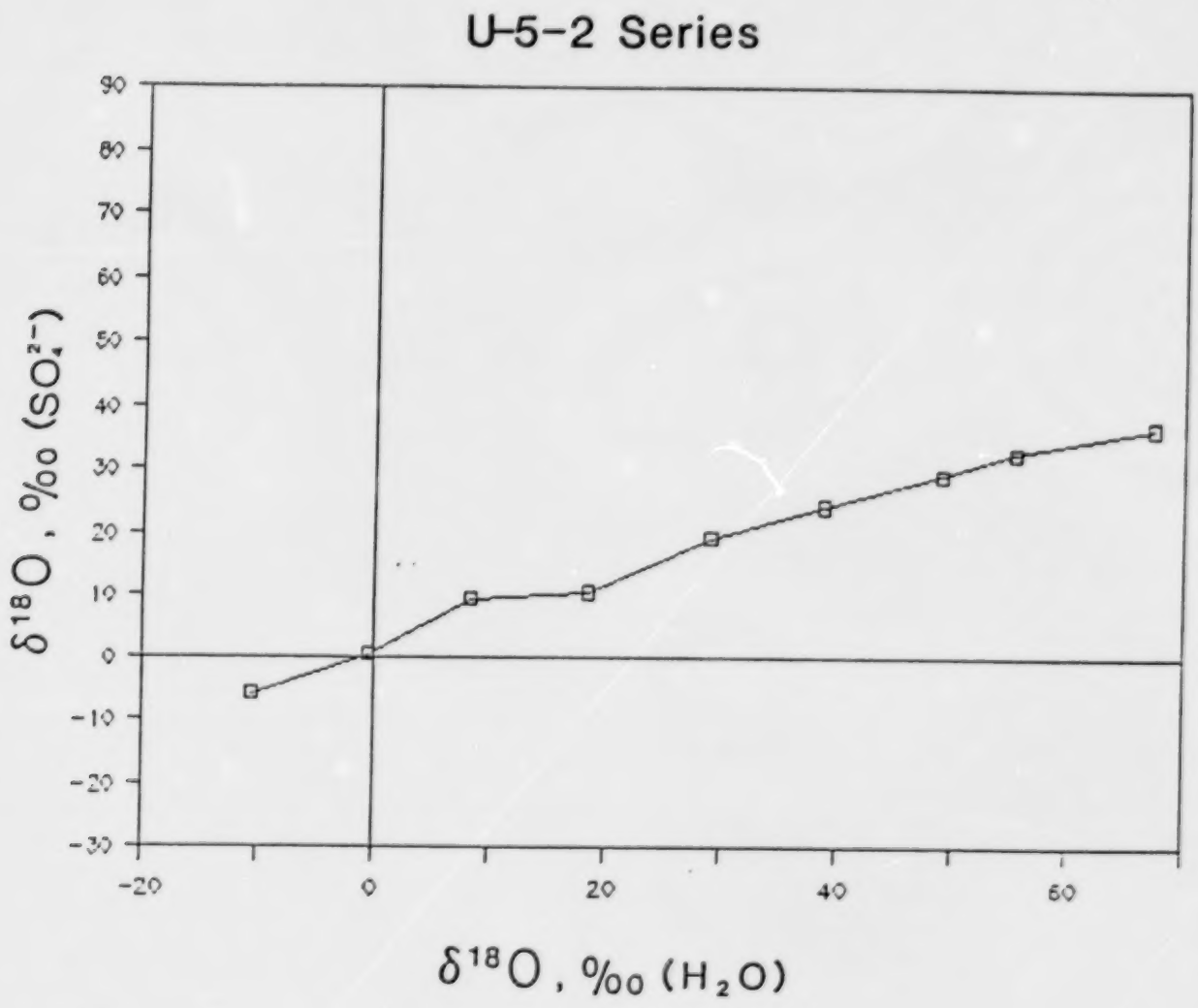

Figure 15 $\delta^{18}O$ of sulphate versus $\delta^{18}O$ of water for pyrite oxidation by T. ferrooxidans (EL-5), 5 ml-atm O_2 excess, 2 weeks.

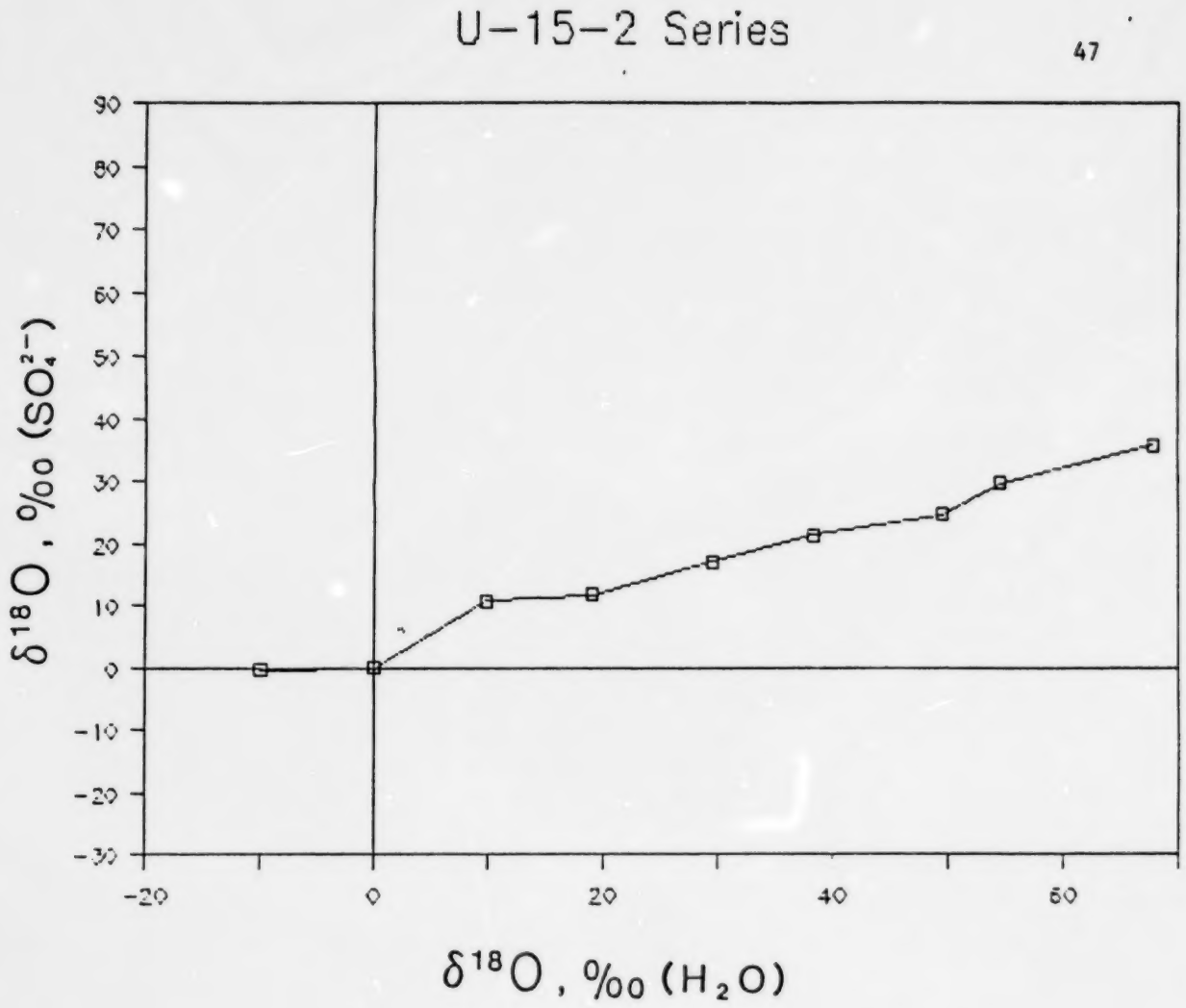

Figure 16 $\delta^{18}O$ of sulphate versus $\delta^{18}O$ of water for pyrite oxidation by T. ferrooxidans (EL-5), 15 ml-atm O_2 excess, 2 weeks.

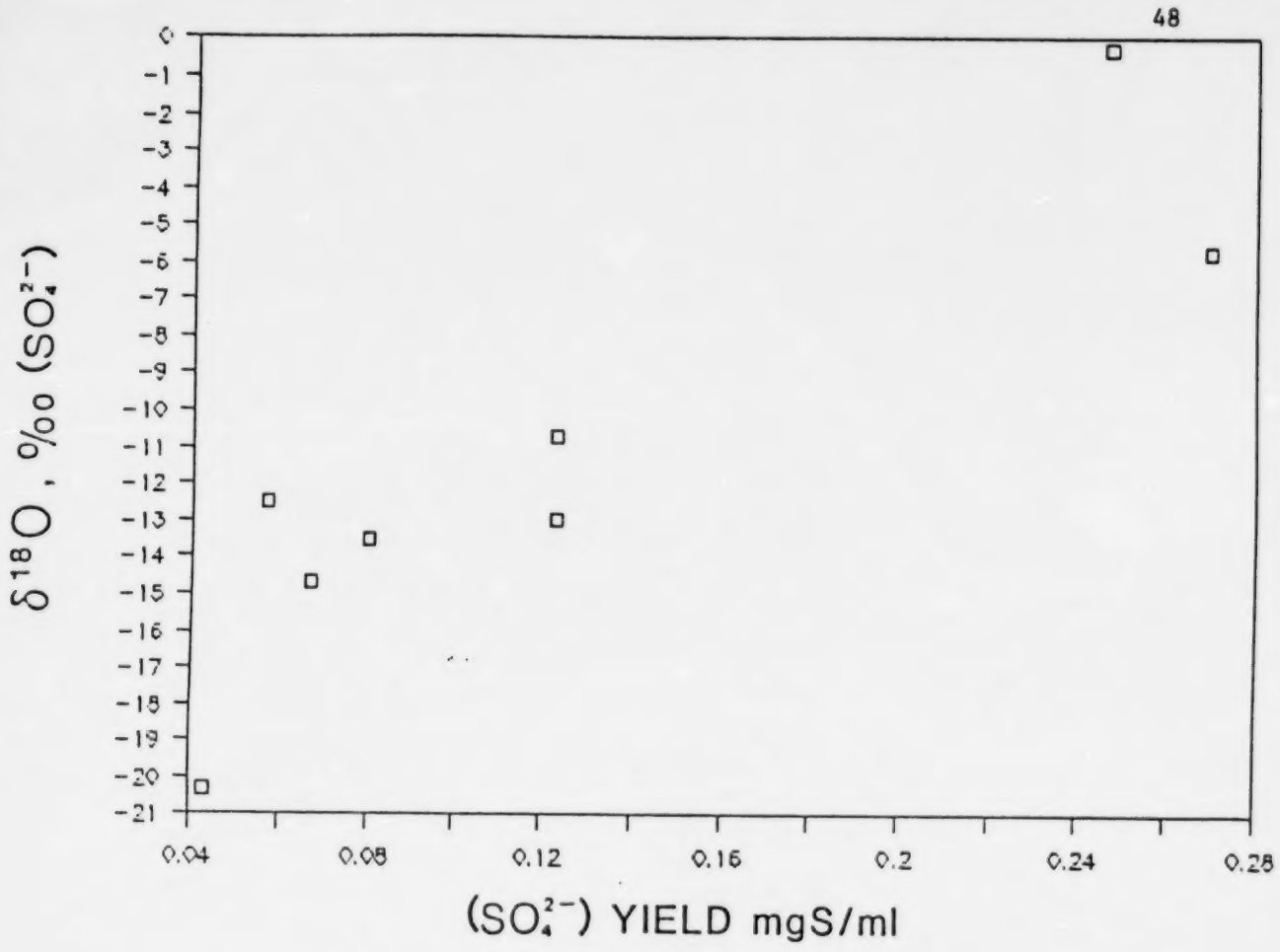

Figure 17 $\delta^{18}O$ (SO_4^{2-}) versus SO_4^{2-} yield during oxidation by T. ferrooxidans, sealed container, tap water.

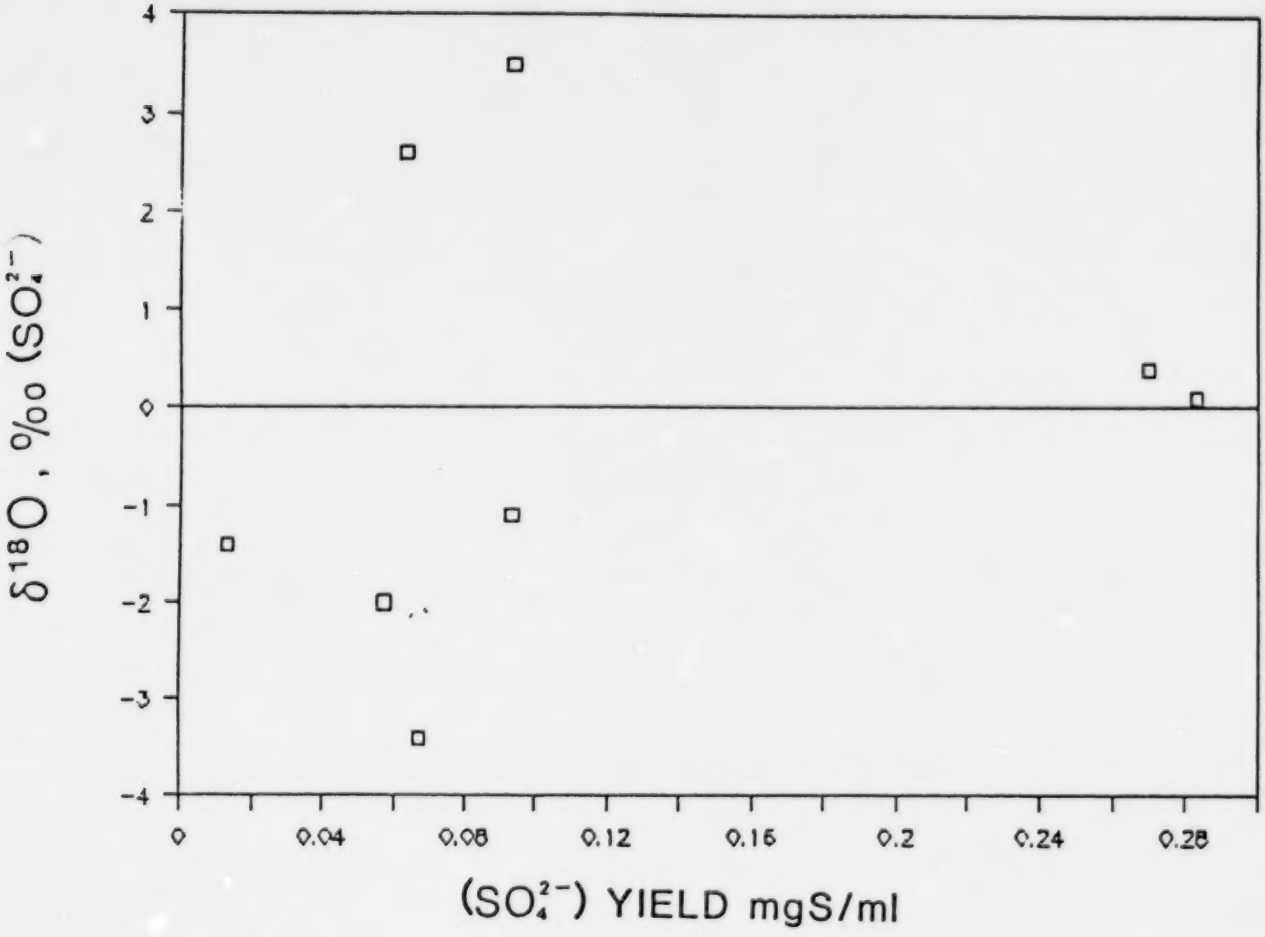

Figure 18 $\delta^{18}O$ (SO_4^{2-}) versus SO_4^{2-} yield during oxidation by T. ferrooxidans, sealed container, 0.01 ml $H_2{}^{18}O$ added.

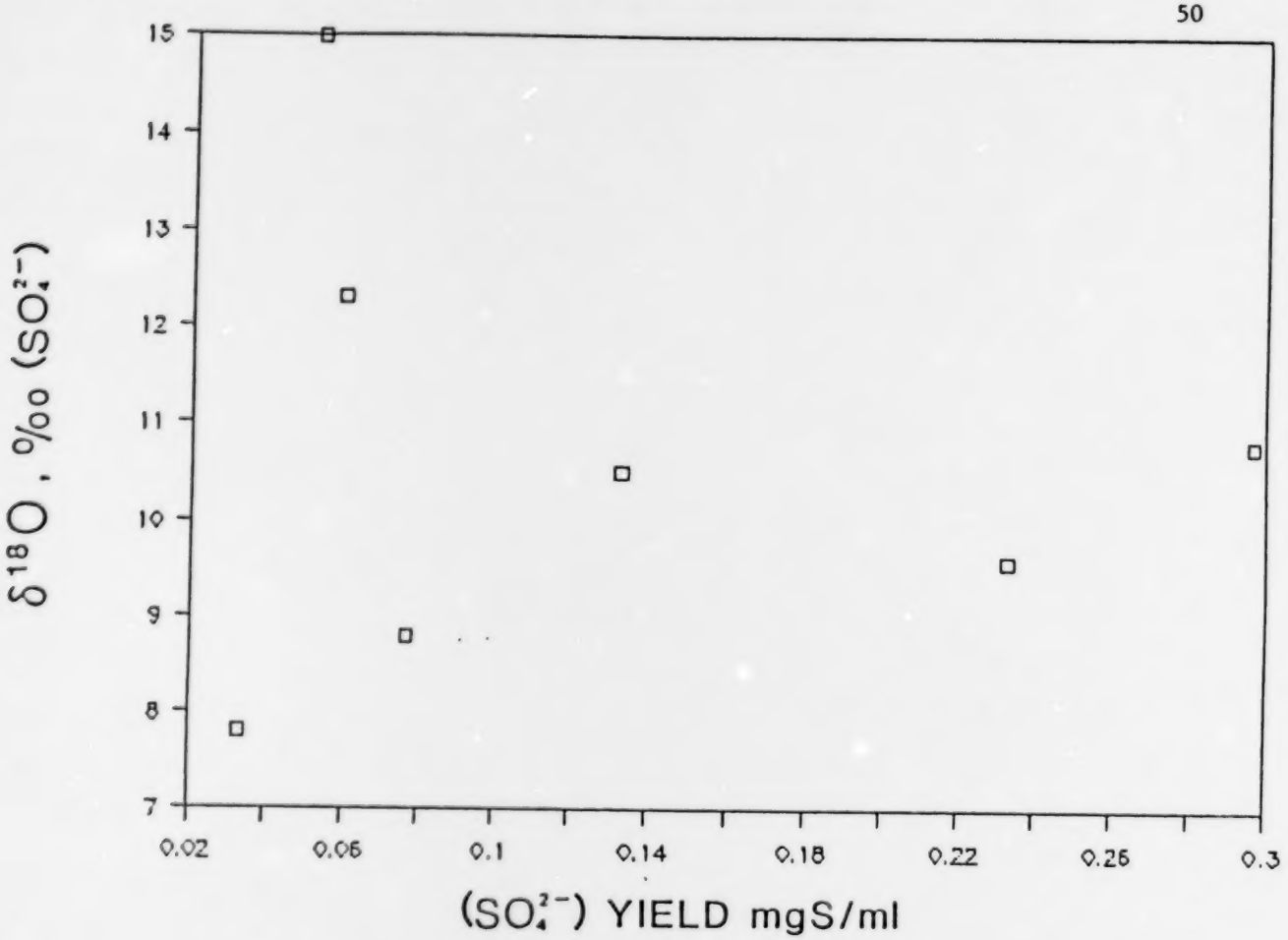

Figure 19 $\delta^{18}O$ (SO_4^{2-}) versus SO_4^{2-} yield during oxidation by T. ferrooxidans, sealed container, 0.02 ml $H_2{}^{18}O$ added.

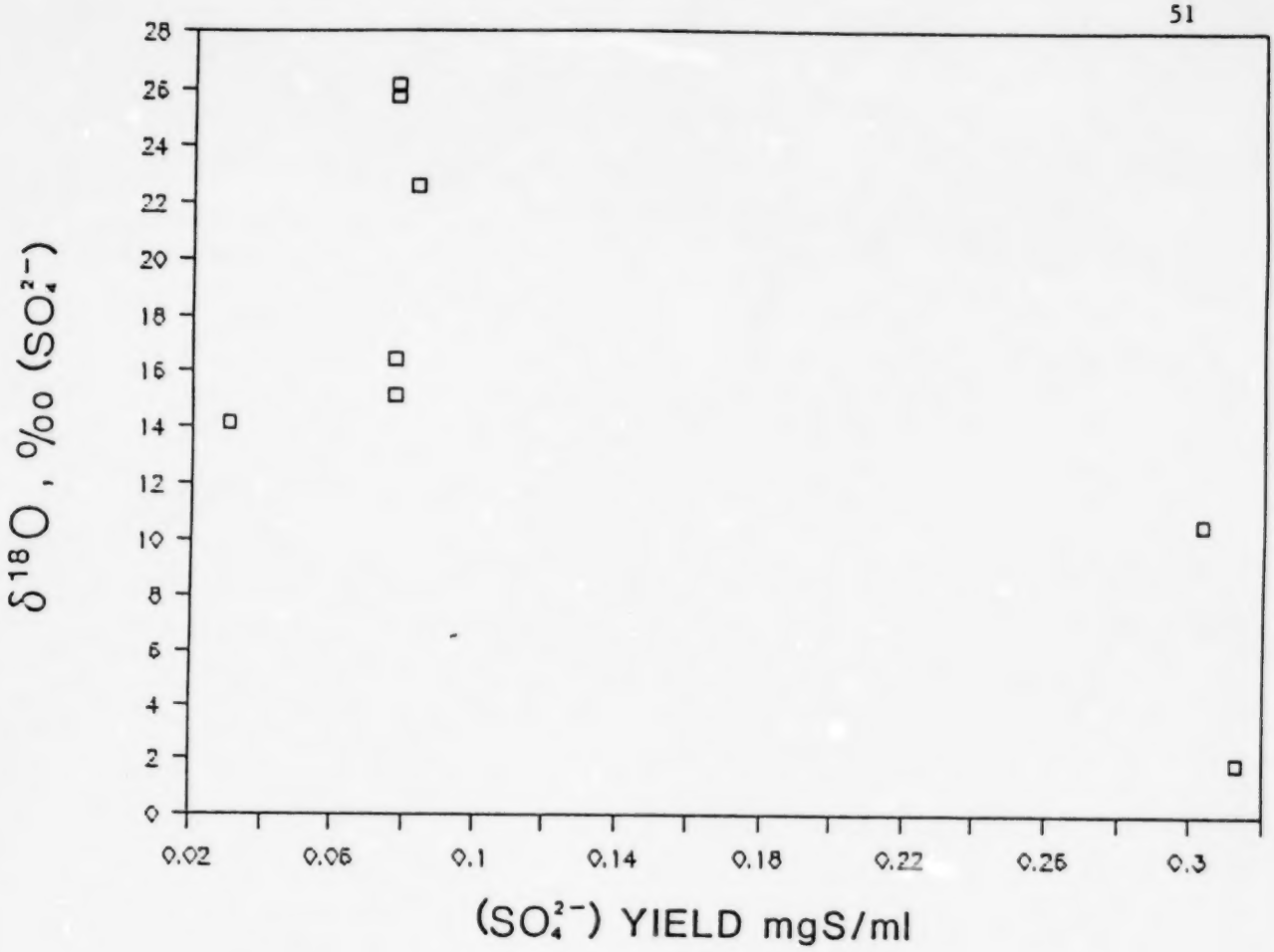

Figure 20 $\delta^{18}O$ (SO_4^{2-}) versus SO_4^{2-} yield during oxidation by T. ferrooxidans, sealed container, 0.03 ml $H_2{}^{18}O$ added.

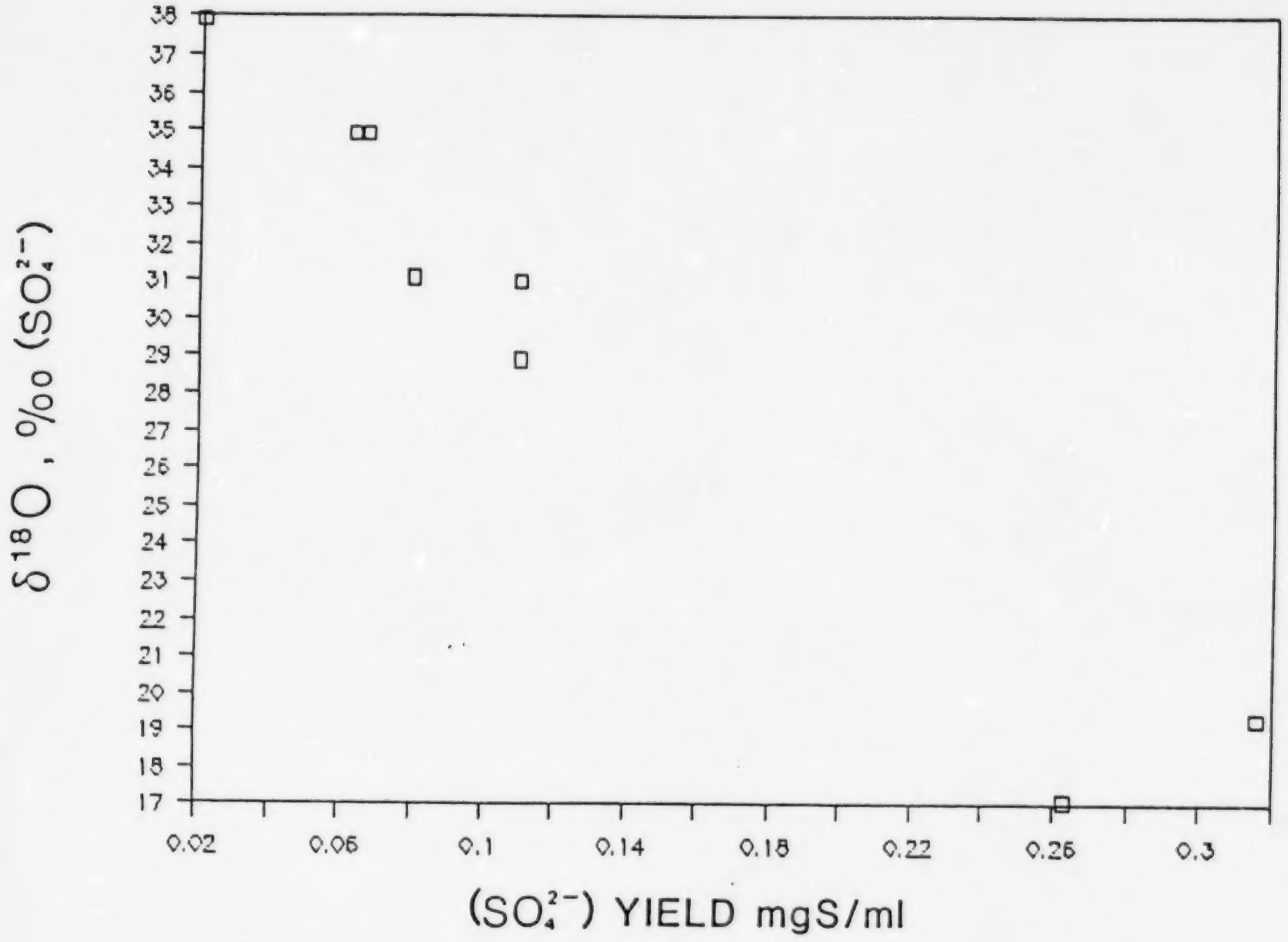

Figure 21 $\delta^{18}O$ (SO_4^{2-}) versus SO_4^{2-} yield during oxidation by T. ferrooxidans, sealed container, 0.04 ml $H_2{}^{18}O$ added.

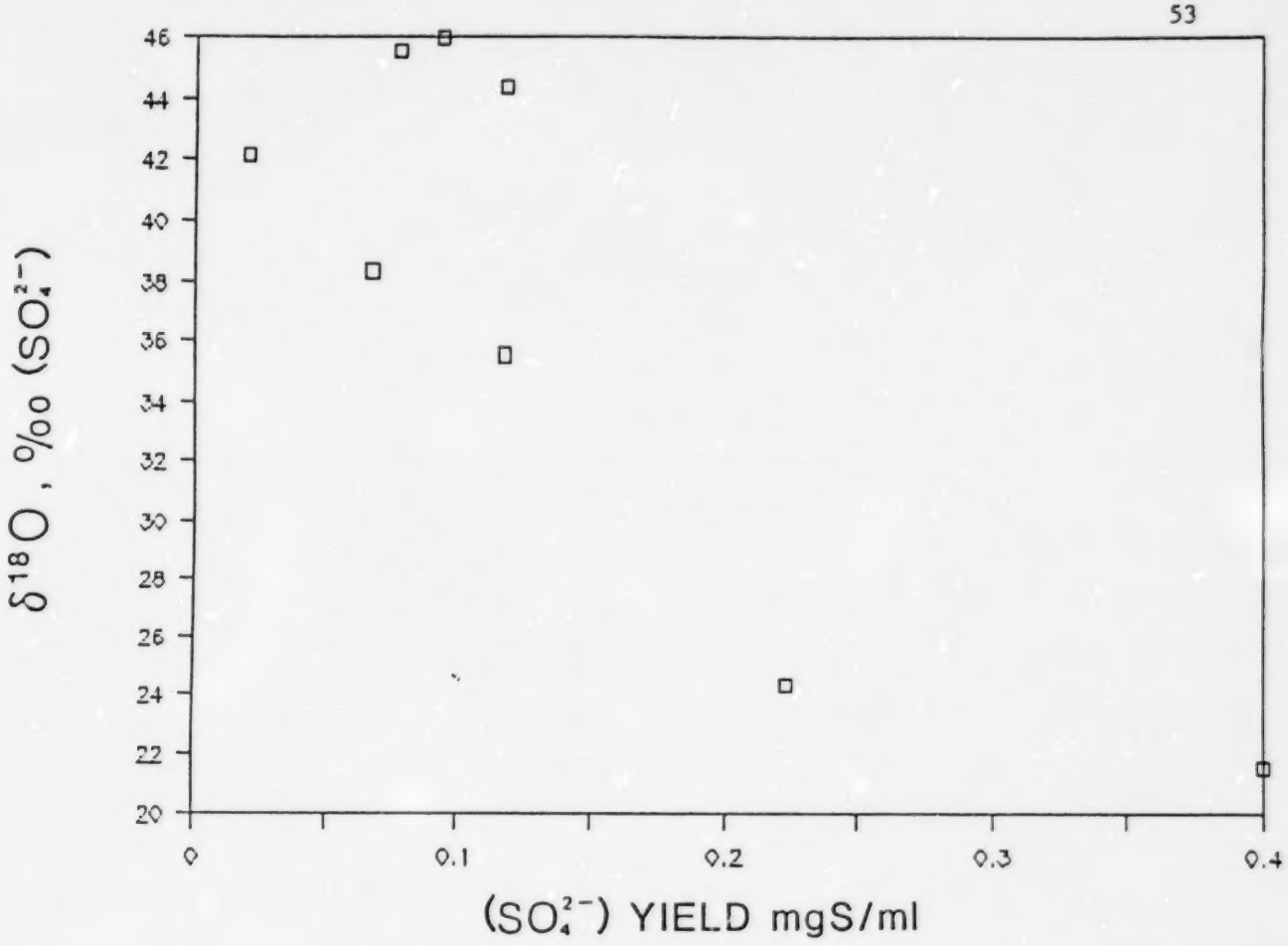

Figure 22 $\delta^{18}O$ (SO_4^{2-}) versus SO_4^{2-} yield during oxidation by T. ferrooxidans, sealed container, 0.05 ml $H_2{}^{18}O$ added.

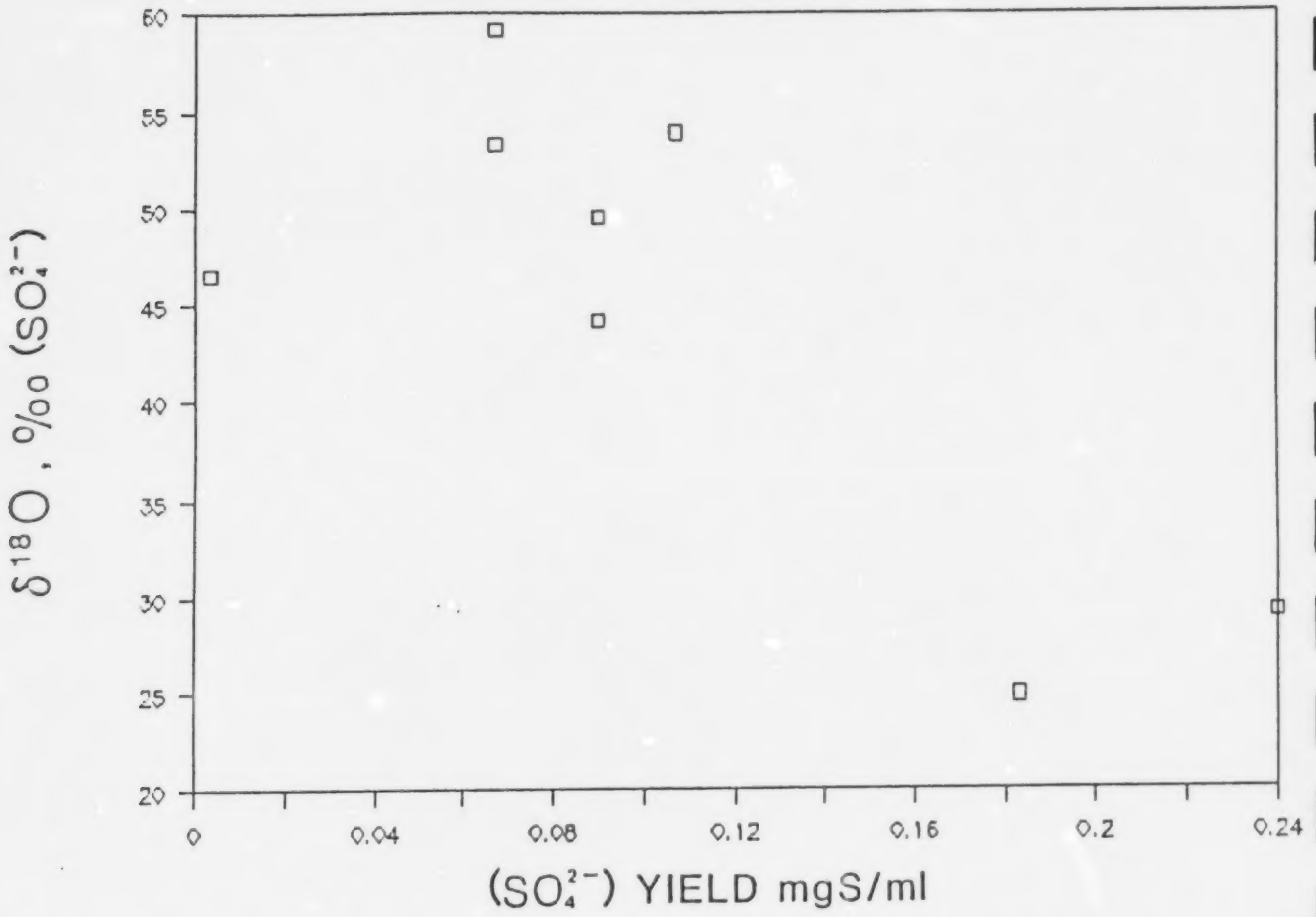

Figure 23 $\delta^{18}O$ (SO_4^{2-}) versus SO_4^{2-} yield during oxidation by T. ferrooxidans, sealed container, 0.06 ml $H_2{}^{18}O$ added.

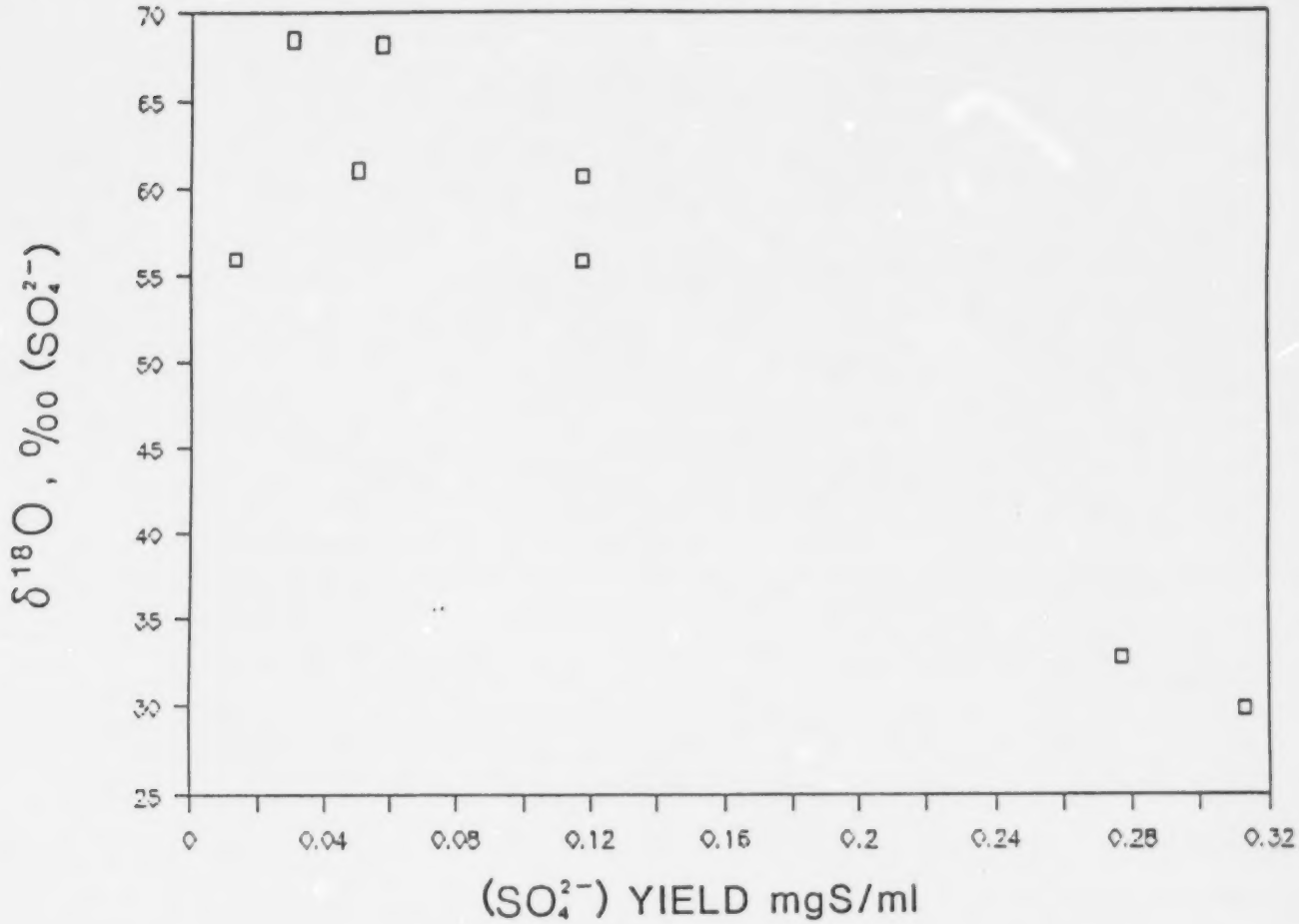

Figure 24 $\delta^{18}O$ (SO_4^{2-}) versus SO_4^{2-} yield during oxidation by T. ferrooxidans, sealed container, 0.07 ml $H_2{}^{18}O$ added.

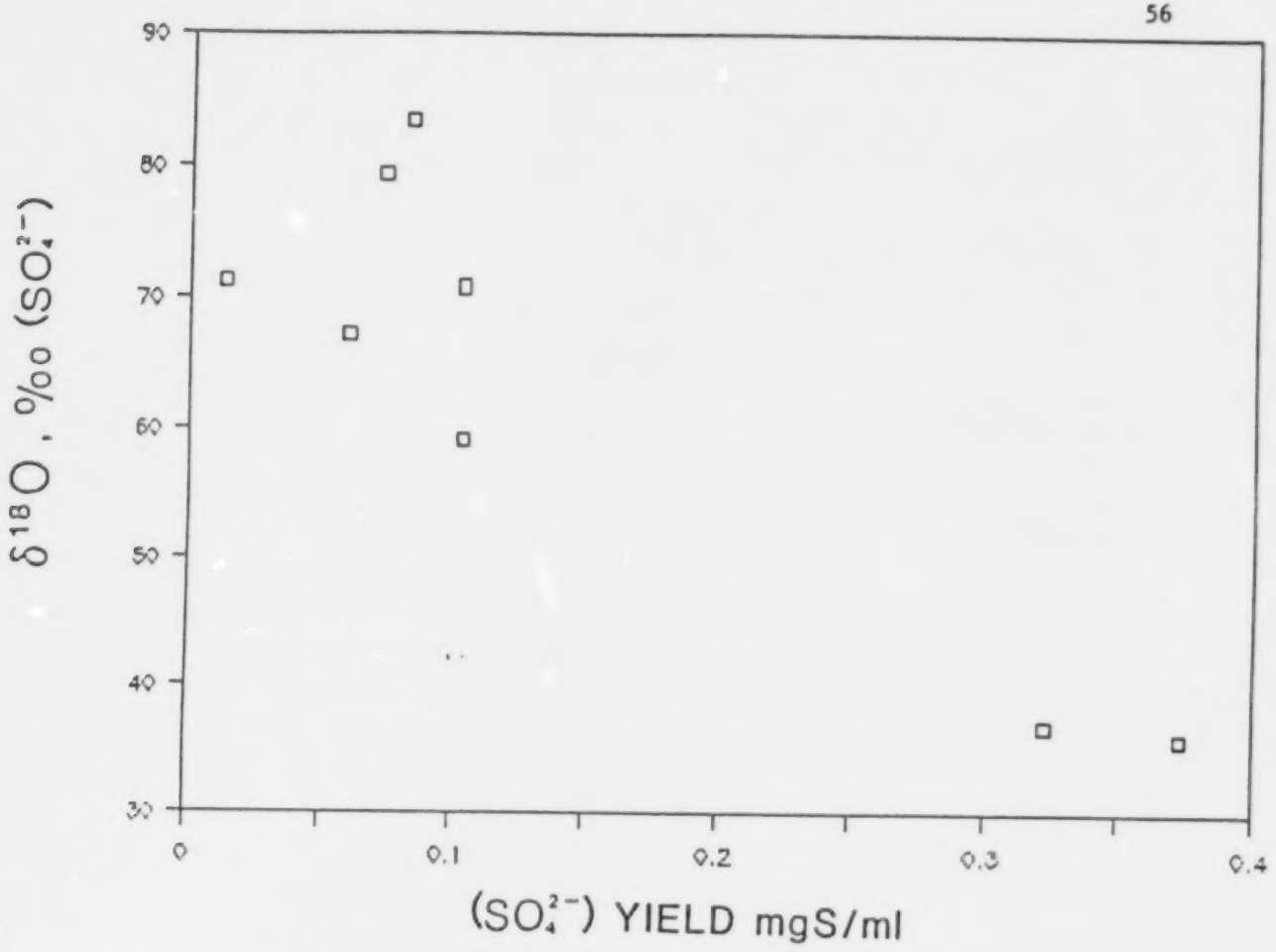

Figure 25 $\delta^{18}O$ (SO_4^{2-}) versus SO_4^{2-} yield during oxidation by T. ferrooxidans, sealed container, 0.08 ml $H_2{}^{18}O$ added.

V SERIES EXPERIMENTS: Thiosulphate to sulphite conversion with rhodanese.

Thiosulphate was converted to sulphite according to the reaction

$$SSO_3^{2-} + CN \xrightarrow{\text{Rhodanese}} SCN + SO_3^{2-}$$

Rhodanese (EC 2.8.1.1; 10-20 units/mg) was obtained from Sigma Chemical Co., St. Louis, Missouri.

Nine 250 ml Erlenmeyer flasks containing 5 ml of $Na_2S_2O_3$ (25 µmoles), 5 ml of rhodanese solution (0.75 mg enzyme), and 5 ml of KCN solution (25 µmoles) were sealed with rubber septa. All of these solutions were dissolved in 0.1M TRIS buffer, pH 8.5. Varying amounts of $^{18}O_2$ were injected into the headspaces of the flasks. The reaction was begun by the addition of the KCN solution. An identical series of controls without enzyme were also set up. The flasks were incubated for 24 h at 23°C. Thiocyanate concentrations were determined by mixing 0.2 ml of the sample with 4.3 ml double distilled water and 0.5 ml of 20% $FeNO_3$ dissolved in 3.0 N HNO_3. The optical density of the solution was measured at 460 nm. To 5 ml of the reaction mixture was added 0.1 ml of the ferric nitrate solution to stop the reaction. This sample was saved for ^{18}O analysis.

The data are given in Table XIV.

It is noted that the reaction did not proceed in the absence of rhodanese and exceeded 80 percent when rhodanese was present.

It is also seen that the $\delta^{18}O$ value of the water remained constant signifying that no detectable oxygen atom exchange between H_2O and O_2 occurred. Since the O_2 had large ^{18}O enrichments, exchange would have been readily detected.

TABLE XIV V-SERIES: CONVERSION OF THIOSULPHATE TO SULPHITE

SAMPLE	$^{18}O_2$ added (ml-atm)	% reaction	$\delta^{18}O$ (H_2O)
with enzyme			
V-1	0	81	-11.1
V-2	1	83	-11.0
V-3	2	87	-10.4
V-4	3	80	-10.7
V-5	4	87	-10.6
V-6	5	88	-11.1
V-7	6	87	-10.0
V-8	7	92	-10.8
V-9	8	81	-11.9
without enzyme			
V-10	0	0	-11.4
V-11	1	0	-10.8
V-12	2	0	-11.7
V-13	3	0	-10.4
V-14	4	0	-11.4
V-15	5	0	-10.1
V-16	6	0	-12.7
V-17	7	0	-10.8
V-18	8	0	-11.6

SERIES W AND X: Oxidation of SO_3^{2-}.

MATERIALS AND METHODS

The potential for sulphite oxidase mediated ^{18}O exchange was examined by two techniques (1) incubation in water of varying $H_2{}^{18}O$ composition (Series W) and (2) incubation in sealed 250 ml Erlenmeyer flasks containing varying amounts of $^{18}O_2$ (Series X). The sulphite oxidase assay was a variation of that used by Lyric and Suzuki (1970). Sulphite oxidase (EC 1.8.3.1; 70 units/mg protein), and cytochrome C (horse heart, type III) were obtained from Sigma Chemical Co., St. Louis, Missouri. In the $^{18}O_2$ experiment (Series X), each flask contained 25 ml of cytochrome C solution (6.25 mg), 50 ml of Na_2SO_3 solution (500 µmoles) and 5 ml of sulphite oxidase solution (12.5 units). The reaction mixtures were made up to 100 ml volume by the addition of 10 mM TRIS buffer, pH 8.0. The substrates and enzymes were also made up in 10mM TRIS buffer, pH 8.0. The flasks were then sealed with rubber septa. Varying amounts (1 to 8 ml) of $^{18}O_2$ were injected into the headspaces of the flasks. The same procedure was repeated under ambient atmosphere with the W series of flasks in which the $H_2{}^{18}O$ content was varied by the addition of different quantities of 10% enriched $H_2{}^{18}O$. The reactions were initiated by the addition of enzyme. For both of these experiments, a series of control flasks with no enzymes were also run. The flasks were incubated for 20 h at 23°C. When the experiment was terminated, 90 ml of the solution was used for the collection of sulphate and 10 ml

was acidified to pH 2.0 and saved for $H_2{}^{18}O$ analysis.

Data for the W-series experiments are given in Table XV and Figures 26 and 27. Figure 26 is essentially a check on the isotopic balance of water i.e. the $\delta^{18}O$ value of the water should increase in an approximately linear fashion with the amount of added $H_2{}^{18}O$.

In Figure 27, it is seen that oxygen was incorporated from H_2O into the $SO_4{}^{2-}$ with or without the oxidase enzyme present. However, the $SO_4{}^{2-}$ yields with the enzyme were an order of magnitude higher than without (Table XV). The linear fits in Figure 27 are

With Enzyme

$$\delta^{18}O\ (SO_4{}^{2-}) = (0.56\pm0.02)\delta^{18}O(H_2O) + (8.3\pm4.8)$$

Without Enzyme

$$\delta^{18}O\ (SO_4{}^{2-}) = (0.58\pm0.05)\delta^{18}O(H_2O) + (13.1\pm8.7)$$

This agreement means that the oxygenase was not influential in determining the relationship between the $\delta^{18}O$ values of $SO_4{}^{2-}$ and water.

It also seems relevant that the average slope of 0.57 is in the range found in the bacterial FeS_2 oxidation experiments. It is lower than the value of 0.7 realized in experiments open to the atmosphere and just slightly higher than the values (0.55, 0.49) obtained in the closed vessel U-series experiment of two weeks duration (Table XIII).

The data for the X-series of experiments are given in Table XVI and Figure 28. It is seen that oxygen from O_2 is incorporated into SO_4^{2-} during SO_3^{2-} oxidation with or without oxidase enzyme present.

The SO_4^{2-} yields with oxidase enzyme present, were an order of magnitude higher than without enzyme (Table XVI).

The ^{18}O enrichments in SO_4^{2-} were about a factor of 2 higher when enzyme was present than when oxidase was absent. This is a significant observation.

The $\delta^{18}O$ value of water remained constant regardless of the amount of added $^{18}O_2$. It is noted that at maximum $^{18}O_2$ addition, the number of ^{18}O atoms added corresponded to about 43 percent of those present in the initial water. Therefore it must be concluded that oxygen atoms did not exchange between O_2 and H_2O.

TABLE XV W-SERIES: SULPHITE OXIDATION EXPERIMENTS WITH VARIOUS ADDITIONS OF $H_2{}^{18}O$

SAMPLE	$H_2{}^{18}O$ ADDITION ml/100 ml	$BaSO_4$ (mgS)	$\delta^{18}O$ (‰ SMOW) $SO_4{}^{2-}$	H_2O
WITH ENZYME				
W-1	0.00	6.80	4.50	-11.10
W-2	0.05	6.70	18.40	15.10
W-3	0.10	6.50	33.40	44.60
W-4	0.15	6.90	52.20	78.80
W-5	0.20	5.00	55.80	92.10
W-6	0.25	6.40	80.90	127.70
W-7	0.30	6.40	81.90	147.30
W-8	0.35	6.90	112.80	175.30
W-9	0.40	6.80	123.40	198.90
WITHOUT ENZYME				
W-10	0.00	0.80	15.50	-10.40
W-11	0.05	0.70	18.60	20.60
W-12	0.10	0.50	NE	41.00
W-13	0.15	0.80	46.00	75.40
W-14	0.20	0.80	71.80	95.80
W-15	0.25	0.40	NE	117.10
W-16	0.30	0.80	100.00	146.50
W-17	0.35	1.70	121.90	170.60
W-18	0.40	0.50	123.40	200.70

TABLE XVI X-SERIES: SULPHITE OXIDATION EXPERIMENTS WITH VARIOUS ADDITIONS OF $^{18}O_2$

SAMPLE	$^{18}O_2$ ADDITION ml/100 ml	$BaSO_4$ (mgS)	$\delta^{18}O$ (°/₀₀ SMOW) SO_4^{2-}	H_2O
WITH OXIDASE ENZYME				
X-1	0.00	7.50	1.00	-10.80
X-2	1.00	7.50	1494.00	-10.30
X-3	2.00	8.00	4189.00	-10.80
X-4	3.00	7.80	6640.00	-10.70
X-5	4.00	7.30	8354.00	-10.00
X-6	5.00	7.90	8844.00	-10.40
X-7	6.00	7.80	11294.00	-10.10
X-8	7.00	7.90	13254.00	-10.80
X-9	8.00	8.60	11539.00	-10.90
WITHOUT ENZYME				
X-10	0.00	0.50	NE	-10.90
X-11	1.00	0.40	931.00	-10.00
X-12	2.00	0.50	2744.00	-10.60
X-13	3.00	0.30	NE	-10.80
X-14	4.00	1.00	2976.00	-11.20
X-15	5.00	0.40	3577.00	-11.20
X-16	6.00	0.50	5023.00	-10.90
X-17	7.00	0.50	5317.00	-11.10
X-18	8.00	0.50	6199.00	-10.70

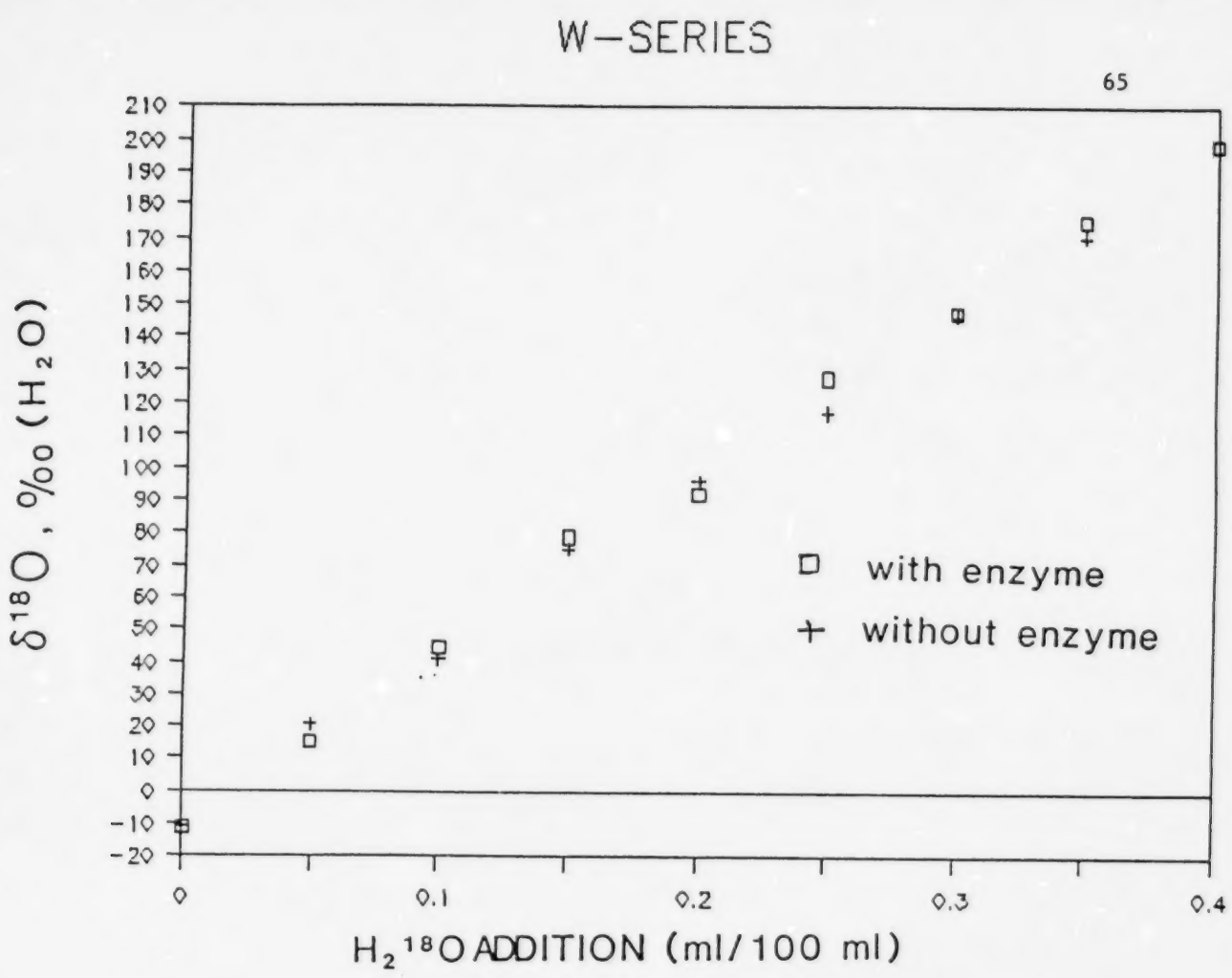

Figure 26 $\delta^{18}O$ of H_2O versus $H_2{}^{18}O$ ADDITION for SO_3^{2-} oxidation experiment.

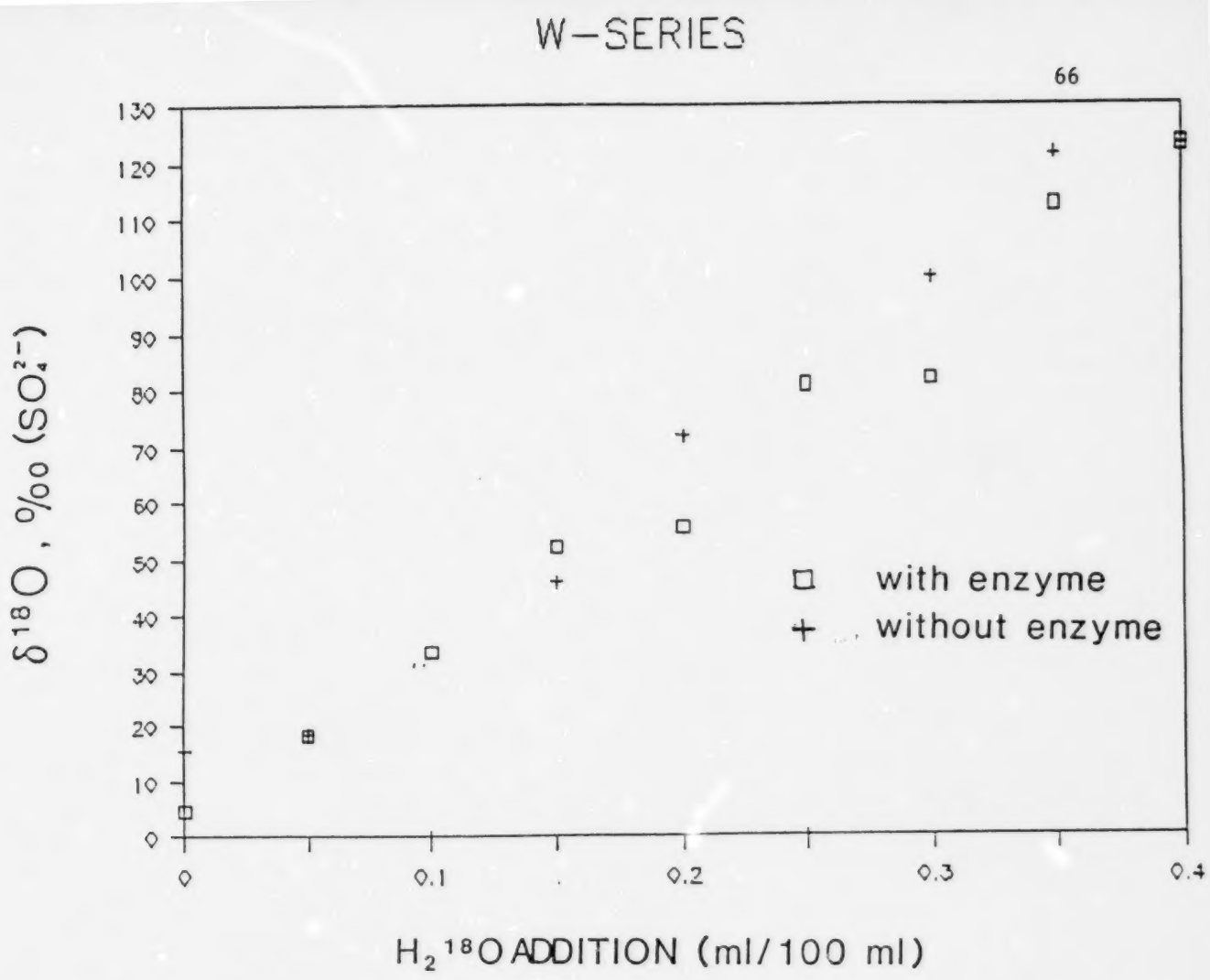

Figure 27 Dependence of $\delta^{18}O$ value of SO_4^{2-} on $H_2{}^{18}O$ addition during SO_3^{2-} oxidation.

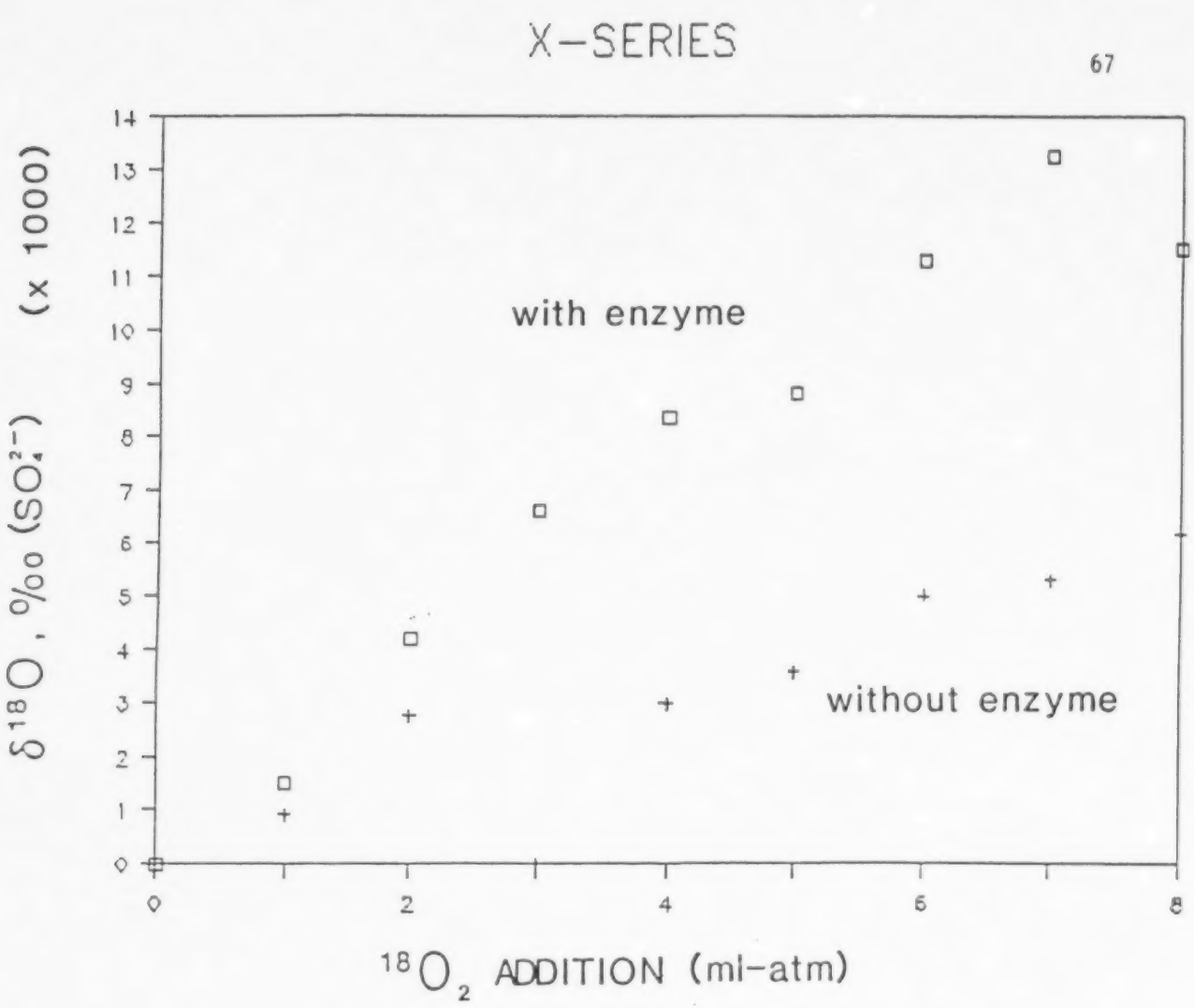

Figure 28 $\delta^{18}O$ of SO_4^{2-} versus $^{18}O_2$ addition for oxidation of SO_3^{2-}.

SUMMARY AND RECOMMENDATIONS

The S-series of experiments in which pentlandite was oxidized yielded results similar to previous studies. The slope of the $\delta^{18}O$ (SO_4^{2-}) versus $\delta H_2{}^{18}O$ line was found to be 0.74 as found for FeS_2 and pentlandite in other experiments open to the atmosphere. However, the remaining series of experiments were unlike those reported in the literature and "landmark" observations have enhanced our understanding of bacterial mineral sulphide oxidation.

It was shown clearly in the R-series of experiments the O_2 which does not normally exchange oxygen atoms with H_2O, does so during bacterial oxidation of pentlandite. This observation has profound geochemical significance since H_2O and O_2 constitute major terrestrial reservoirs of oxygen. This exchange also bears directly on the bacterial oxidation mechanism. It is known that SO_4^{2-} does not readily exchange oxygen atoms with H_2O (Miyoshi et al., 1984). Therefore, H_2O must be capable of exchanging oxygen atoms with an intermediate in the reaction which acquired oxygen from O_2. However, since the O_2 had "lost" its ^{18}O enrichment during the sulphide oxidation, it also follows that O_2 can exchange with intermediates in the reaction. It is noted that O_2 and H_2O need not exchange oxygen isotopes with the same intermediate to have effectively exchanged oxygen isotopes with each other.

Although the U-series of experiments represent the classic case of providing more questions than answers, it

must be deduced that (1) the oxidation rate increased slightly with higher O_2 pressure (2) O_2 pressure influences the oxygen isotope composition of SO_4^{2-} during bacterial oxidation, and (3) there are significant kinetic isotope effects during O_2 incorporation. The findings of the closed container experiments are relevant to O_2 limited subsurface environments.

Experiment series V, W, and X constitute studies of parts of the overall mechanism. In such studies, there is the question as to whether behavior of isolated reaction steps will be the same as when they are combined with other steps and the participation of intact bacteria. However, information can be obtained from studies of individual steps and there are not many alternate methods.

It was found that during the rhodanese catalysed conversion of SSO_3^{2-} to SO_3^{2-}, oxygen atoms were not transferred from O_2 to H_2O (V-series experiments).

During oxidation of SO_3^{2-} to SO_4^{2-}, oxygen was incorporated from H_2O into SO_4^{2-} with or without the oxidase enzyme present. The slope for the line of $\delta^{18}O$ values of the SO_4^{2-} versus those of the H_2O, 0.57, is close to that realized in bacterial mineral sulphide oxidation. In other words the SO_3^{2-} oxidation step may very well be the dominant determiner of the oxygen isotope composition of the SO_4^{2-}.

For a half century, it has been known that oxygen isotope exchange between SO_3^{2-} and H_2O may proceed quickly but it is inhibited by high pH (Hall and Alexander, 1940).

Unfortunately in the current studies, SO_3^{2-} was not isolated to see if it had isotopically equilibrated with the water in which case there would have been approximately a constant difference in $\delta^{18}O$ value between the SO_3^{2-} and H_2O. Such a constant difference in $\delta^{18}O$ value between the SO_4^{2-} and H_2O was not observed because of the slope of 0.57 discussed above, rather than unity. Stating it another way, at low $H_2{}^{18}O$ enrichments, the SO_4^{2-} had a higher $\delta^{18}O$ value than the H_2O whereas the opposite case was found at higher $\delta^{18}O$ values for the H_2O. Such behavior cannot arise from solely oxygen isotope exchange.

The X-series of experiments illustrate conclusively the participation of O_2 in oxidizing SO_3^{2-} to SO_4^{2-}. This participation cannot be by exchange with H_2O since it retained its initial $\delta^{18}O$ value. The finding of higher $\delta^{18}O$ values in the SO_4^{2-} when the oxidase enzyme is present is also revealing. It means that the ratio of the oxygen in the SO_4^{2-} derived from O_2 to that from H_2O is higher when oxidase is present. One then has to question why this effect with oxidase was apparently not seen when comparing the $\delta^{18}O$ values of SO_4^{2-} to those of H_2O in the W-series experiment. In fact, the slope of the $\delta^{18}O$ (SO_4^{2-}) versus $\delta^{18}O$ (H_2O) line was slightly lower for enzyme catalysed (0.56) than for enzyme absent (0.58). This is consistent with the results of the X-series experiments, but the standard deviations of the slopes overlap. One must realize that with the constant $\delta^{18}O$ value of +23°/$_{oo}$ for O_2 in the W-series experiment, there was not

sufficient "isotopic leverage" to see the effects of oxygenase altering the ratio of SO_4^{2-} oxygen derived from O_2 and H_2O.

The results of these studies are also relevant to the findings of Mizutani and Rafter (1973). They found that during SO_4^{2-} reduction, the $\delta^{18}O$ value of the "unreacted" SO_4^{2-} was dependent upon that of the water in the medium. They postulated oxygen isotope exchange between H_2O and intermediates in the reaction. Subsequently "back reaction" of the intermediate introduced oxygen from H_2O into the SO_4^{2-}. The W-series of experiments represent an important addition to the argument. It was demonstrated that during SO_3^{2-}oxidation (a possible "back reaction" during SO_4^{2-} reduction) oxygen from the H_2O could be conveyed to SO_4^{2-}. Although exchange of oxygen atoms between H_2O and SO_3^{2-} has been known for many years (Hall and Alexander, 1940), this fact only support part of the postulate of Mizutani and Rafter (1973).

From the viewpoint of better understanding of the mechanism of bacterial sulphide oxidation, there are further desirable experiments.

The effect of O_2 pressure on the oxygen isotope composition of SO_4^{2-} should be examined with a large O_2 reservoir and constant conditions of pressure and carbon source in each experiment.

The effect of oxygenase promoting greater derivation of oxygen from O_2 than from H_2O should be further checked in

experiments using O_2 with a higher $\delta^{18}O$ value and varied $\delta^{18}O$ values for H_2O.

Studies involving intermediate reaction steps should be carried out over at different pH values over which a given enzyme remains stable. This is desirable since it is known that oxygen isotope exchange between H_2O and ions tends to be pH dependent.

One is faced with the justification of further detailed studies of a system aside from elucidating the mechanisms. The understanding of sulphide oxidation in O_2 limiting subsurface systems is of considerable geochemical importance and relates to global cycling of elements and global change. Better understanding of the enzyme mechanisms in mineral sulphide oxidation is not only desirable from the viewpoint of enhancing or retarding the reaction, but the findings should have ramifications in other research fields such as the medical sciences.

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